PROTEIN TARGETING AND TRANSLOCATION ON cpSEC AND DELTA pH PATHWAYS IN CHLOROPLAST THYLAKOIDS

By

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KEY TO ABBREVIATIONS

APDP	N-[4-(p-Azidosalicylamido) butyl]-3 -[2' pyridyldithio] propionamide
BS^3	bis [sulfosuccinimidyl] suberate
CCCP	carbonylcyanide 3-chlorophenylhydrazone
DSS	disuccinimidyl suberate
DTT	dithiothreitol
LTD	lumen targeting domain
LHCP	light-harvesting chlorophyll a/b protein
MBS	m-Maleimidobenzoyl-N-hydroxysuccinimide ester
OE17	the 17-kDa subunit of the oxygen-evolving complex
OE23	the 23-kDa subunit of the oxygen-evolving complex
OE33	the 33-kDa subunit of the oxygen-evolving complex
Pmf	proton motive force
PC	plastocyanin
SE	stromal extract
STD	stroma targeting domain

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Protein transport is required for biogenesis of the chloroplast, the site of photosynthesis. There are at least two pathways for protein transport into the chloroplast thylakoid lumen. One pathway is named the cpSec pathway, another is named the Delta pH pathway. Each pathway is specific for a subset of precursor proteins. I hypothesize that targeting, a specific interaction between the precursor and components of the protein transport machinery, commits precursor proteins to a specific pathway.

I have attempted to describe and characterize the first committed step on the cpSec pathway and to define in general terms the sequences required for commitment. Binding of precursors to the thylakoid membrane appears to be the first committed step. It required stromal protein, of which cpSecA was the essential component, and occurred when transport was inhibited by removing ATP from the assay with apyrase. Subsequent transport of bound precursor required ATP, but was not stimulated by additional cpSecA.

My results show that precursor binding to the membrane results in formation of a large complex that includes precursor, cpSecA, and cpSecY. The complex formation with cpSecA is precursor specific, requiring a cpSec transit peptide and additionally a cpSec-compatible flanking region.

Techniques were developed to dissect protein transport on the Delta pH pathway into binding (targeting) and chase (translocation). A newly constructed precursor protein tOE17 that uses the Delta pH pathway, bound to the membrane in the absence of the ΔpH. Binding is productive because bound precursor was transported into the lumen when the ΔpH was restored. Productive binding was competed by saturating amounts of a Delta pH pathway precursor protein, suggesting that precursor tOE17 binds to components of the Delta pH pathway translocation machinery. Consistent with the transport requirement, the ΔpH was the only energy source used for chase. Interestingly, the chase of bound precursor was also competed by over expressed precursor. Antibodies raised to *E. coli*-expressed Hcf106 specifically inhibited tOE17 transport and reduced the level of productive binding. My results suggest that Hcf106 functions in early steps of the transport process, possibly as a receptor.

CHAPTER 1
LITERATURE REVIEW

Although the sun is the primary energy source for all life, it is in chloroplast that the light energy from the sun is converted into biologically usable chemical energy. Apart from photosynthesis, several other important biosynthetic processes also occur in chloroplasts, such as biosynthesis of amino acids, lipids, pigments and plant hormones.

A mature chloroplast can be spatially divided into six compartments, outer envelope, inner envelope, interenvelope space, stroma, thylakoid membrane and thylakoid lumen. The chloroplast is enclosed by the envelopes, inside the chloroplast the thylakoid stacks float in the stroma. The well differentiated chloroplast develops from a small, more or less undifferentiated proplastid. This morphogenetic process is controlled by endogenous and exogenous factors and involves a massive production of proteins that are required for the functions of the chloroplast. The chloroplast is a semi-autonomous organelle. It contains genetic material and has functional systems for both gene transcription and translation. However, its circular DNA molecule contains only about 100 genes, about 69-77 of which encode proteins (Sugiura et al. 1998). More than three fourth of proteins residing in the chloroplasts are nucleus encoded and synthesized in the cytoplasm. These proteins must be imported into the chloroplast from the cytosol. It is obvious that protein import plays an important role in chloroplast biogenesis.

Protein Import into Chloroplast Stroma

Significant advances in understanding chloroplast protein transport began two decades ago when protein import was reconstituted with isolated chloroplasts (Chua and Schmidt 1978, 1979). The *in vitro* or *in organello* transport assay is conducted by mixing radiolabeled precursor protein with purified chloroplasts. Active chloroplasts for protein import are prepared by homogenization of fresh young green tissues followed by Percoll gradient centrifugation and isotonic buffer wash. Radiolabeled precursor proteins are prepared by *in vitro* translation of mRNA with a wheat germ system (Cline et al. 1989) or rabbit reticulocyte system in the presence of one radiolabeled amino acid. Large amounts of precursor protein can be made by expression of the protein in *E. coli* and subsequent purification (Westhoff et al. 1989, Pilon et al. 1990, Waegemann 1990)

Stroma Targeting Domain

A nuclear-encoded chloroplast protein is initially synthesized in the cytosol as a precursor protein that contains a transient transit peptide at its amino terminus (Dobberstein et al. 1977). For stroma-targeted precursor the transit peptide consists of a stromal targeting domain (STD) that governs the import of the precursor across the chloroplast envelope. Once in stroma the precursor stromal targeting domain is cleaved by a processing protease (Robinson and Ellis 1984, Alaut et al. 1989 VanderVere et al. 1995)

Stromal targeting domains range from 30 to 120 amino acid residues and are predicted to have a random coil conformation (von Heijne and Nishikawa 1991). Although the stromal targeting domains from different precursors are interchangeable for

targeting passenger proteins across the chloroplast envelope there are no conserved sequence blocks among the stromal targeting domains. However stromal targeting domains are characterized to share three compositional motifs a 10-15 residue N proximal portion without charged residues, glycine or proline; a variable middle region that lacks negative charged residues and is rich in hydroxylated amino acids (serine and threonine), and a C-terminal region that contains a loosely conserved sequence (Ile/Val-X-Ala/Cys*Ala) for proteolytic processing (von Heijne et al. 1989 de Boer and Weisbeek 1991)

The Import Process

Protein import across chloroplast envelope into the stroma is considered to be a three step process (Cline and Henry 1996 Schnell 1998, Soll and Tien 1998) The first is reversible precursor binding to the outer envelope This step does not require any energy The binding is achieved via the interaction of the precursor stromal targeting domain with proteinaceous receptors and possibly polar lipids at the surface of outer membrane (Cline et al. 1985) The translocon at the outer chloroplastic envelope membrane (Toc complex) contains three integral membrane proteins. Toc34 Toc86 and Toc75 Two lines of evidences suggest that Toc86 and Toc34 function as primary receptors first, cross-linking studies indicate that Toc86 and Toc34 are contact with the precursor during the initial binding (Perry and Keegstra 1994, Ma et al 1996, Kouranov and Schnell 1997), second, anti Toc86 IgGs block the binding of precursor to the envelope (Hirsch et al. 1994).

In the second step of import, the precursor inserts across the outer envelope, resulting in close physical proximity to Toc75 (Perry and Keegstra 1994, Ma et al. 1996). Toc75 is deeply embedded in the outer envelope (Schnell et al. 1994, Tranel et al. 1995). Analysis of the Toc75 sequence indicates that it may traverse the membrane with 16 hydrophobic β-strands. Toc75 is therefore proposed to function as a protein translocation channel and this was verified *in vitro* by studies using expressed Toc75 reconstituted into liposomes (Hinnah et al. 1997). Unlike the first step of import in which the binding is energy independent and reversible, the second step requires the hydrolysis of both ATP and GTP (less than 100 μM) and results in tight association of the precursor with the Toc complex (Kessler et al. 1994, Olsen and Keegstra 1992, Ma et al. 1996). Precursor insertion across the outer envelope also brings the transit peptide (stromal targeting domain) near to the translocon at the inner chloroplastic envelope membrane (Tic complex) (Schnell and Blobel 1993). In their *in vitro* binding assay, Friedman and Keegstra (1989) and Schnell and Blobel (1993) estimated that there are about 1500-3000 import sites per chloroplast. However, immunogold labeling of Toc34 in cryosubstituted thin sectioned chloroplasts suggested that there were at least 15000-20000 import sites (Morin and Soll 1997). The difference indicates that in the isolated chloroplasts only a small percentage of import sites are functional for precursor binding or import, implying that only receptors in a certain state can be used. Both Toc86 and Toc34 are GTP-binding proteins (Kessler et al. 1994). It is possible that GTP status may determine receptor state.

Several proteins have been identified as components of the translocon at the inner chloroplastic envelope. They are Tic14, Tic21, Tic22, Tic55, Tic110 etc. Tic14, Tic21 and Tic22 were identified by cross-linking to a precursor protein (Ma et al. 1996,

Kouranov and Schnell 1997). Tic22 is peripherally localized at the outface of the inner envelope and considered to be an inner envelope receptor. Tic110 was found associated with two stroma chaperones, cpn60 and ClpC; it may be involved in driving precursor transport or in folding of newly imported proteins in the stroma (Kessler and Blobel 1996). Tic55 was found associated with Tic110 and the Toc components Toc86, Toc34 and Toc75 in both blue-native gel electrophoresis and affinity chromatography assays (Caliebe et al. 1997). Tic55 contains an iron sulfur cofactor. Soll and Tien (1998) hypothesized that Tic55 may act as a regulatory factor by using the iron-sulfur cluster as a redox sensor to influence protein transport.

The third step of import is protein translocation into the stroma. This step requires the cooperation of both Toc and Tic complexes. The precursor is transported across both outer and inner envelopes via the contact sites (Schnell and Blobel 1993). ATP hydrolysis at higher concentration (>100 μM) in the stroma is used as energy source (Pain and Blobel 1987, Theg et al. 1989). The stromal targeting domain is cleaved by a soluble processing peptidase during or immediately following protein import into the stroma, creating a mature stromal protein (Robinson and Ellis 1984, Abad et al. 1989, VanderVere et al. 1995).

Protein Transport into Thylakoids

Proteins destined for the thylakoid lumen have a bipartite transit peptide: the amino-proximal region is a stroma-targeting domain (STD), the STD is followed by a lumen-targeting domain (LTD) (Ko and Cashmore 1989, Hageman et al. 1990). The bipartite nature of the transit peptide was first proposed by Smeekens et al. (1986) based on their

assay with chimeric precursor proteins. The precursor PC transit peptide not only directed the precursor into the chloroplast stroma but also targeted the mature PC into its subsequent localization. Import of the precursor across the chloroplast envelope is governed by the STD of the transit peptide and the import process is the same as described above for a nucleus-encoded stromal protein. The STD is cleaved by a processing protease in the stroma, exposing the LTD and giving rise to an intermediate precursor protein. The LTD further directs transport into the thylakoid lumen where the LTD is removed by a second processing protease, thereby producing the mature protein (Halpin et al. 1989).

In vivo and *in organello*, the precursor imports across the chloroplast envelope first and then transports into the thylakoid lumen. However, import and transport are independent processes. With *in vitro* assays, precursor can be directly transported into the thylakoids bypassing the import step (Cline 1986, Kirwin et al. 1989). A stromal intermediate precursor was accumulated when thylakoid transport was inhibited during a chloroplast import assay (Cline et al. 1989). The intermediate was in a productive state and it could be chased into the thylakoids once the transport inhibition was removed (Cline et al. 1993). The plastid-encoded thylakoid protein cytochrome *f* is synthesized with only a LTD, its transport should not have any relation with the import process (Nohara et al. 1996, Zak et al. 1997).

Actually, full-length precursor with both STD and LTD are efficient substrates for transport into isolated thylakoids (Robinson and Klösgen 1994). This implies that thylakoid protein transport employs a loop mechanism similar to that used by the *E. coli* Sec system and the endoplasmic reticulum transport system (Shaw et al. 1988, Kuhn et

al 1994) This hypothesis was clearly proven by Fincher et al. (1998) The topology of precursor insertion into the thylakoid membrane was investigated with a fusion protein comprising a large polypeptide domain fused to the amino terminus of pOE17 While the mature OE17 was transported to the thylakoid lumen, the amino terminus, including the pOE17 transit peptide, remained on the *cis* side of the thylakoid membrane

Although there appears to be one major pathway for protein import across the chloroplast envelope, there are at least four pathways for protein transport into the thylakoid lumen or integration in the thylakoid membrane (Figure 1-1) Multiple pathways for thylakoid protein transport were first recognized by the discovery that different subgroups of precursors had different energy and soluble protein factor requirements for translocation (Cline et al. 1992) The existence of pathway specific translocation machinery was further demonstrated by precursor competition studies (Cline et al. 1993) For example, saturating concentration of hOE23 inhibited transport of OE17 OE23 and PSIIT but was without effect on transport of OE33 and PC

One of the thylakoid transport pathways is called the cpSec pathway since this pathway employs a chloroplast homologue of the bacterial SecA protein. A second pathway is named the Delta pH pathway because in this pathway, the ΔpH across the thylakoid membrane is the only energy requirement for protein transport. The third is the cpSRP pathway and the fourth is the spontaneous pathway Each pathway is specific for a subset of precursor proteins (Cline and Henry 1996 Schnell 1998 Robinson et al. 1998)

In this dissertation, research focuses on protein transport on the cpSec and Delta pH pathways. Literature about protein transport on these two pathways will be reviewed in the following sections.

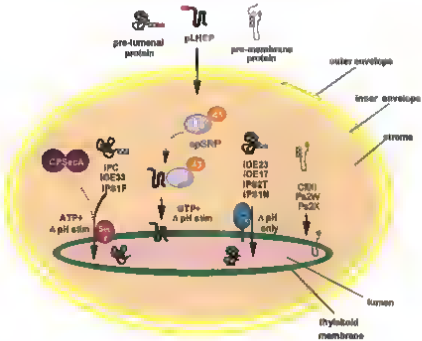


Figure 1-1 Schematic representation of the one general pathway for protein import across chloroplast envelope and four known pathways for protein transport to the thylakoids (From Keegstra and Cline, 1999)

The LTD Structure and Precursor Targeting

The LTD can be divided into four sub-domains based on the nature of the amino acid residues (Cline and Henry 1996): an acidic (A) domain of about 12-15 amino acid residues is on the amino-terminus of the LTD; this is followed by a positively charged (H) domain of about 4-7 amino acid residues, then a hydrophobic (H) domain of 12-18 amino acid, and a C-terminal (C) domain with a relatively conserved motif of Ala-X-Ala for proteolytic processing. Figure 1-2 shows some examples of LTDs that confer either cpSec or Delta pH pathway specifically. Also shown is an artificially produced LTD that confers transport on both pathways.

The N, H and C domains of LTD represent the basic structure of the classical signal peptides that direct proteins to the endoplasmic reticulum or the bacterial plasma membrane. This implies that the LTD is evolutionarily related to these signal peptides and that thylakoid protein transport is conserved from the original bacterial endosymbiont (von Heijne et al. 1989). Additional support for the conservative origin of LTDs came from the fact that LTD is cleaved by a thylakoid luminal processing peptidase (TPP) with identical specificity to a bacterial leader peptidase (LPP), which can carry out faithful processing if the protein is expressed in *E. coli* and exported across bacterial inner membrane (Halpin et al. 1989, Anderson et al. 1991). Thylakoid proteins OE33 and plastocyanin, as well as DT23 with a dual targeting transit peptides, were exported to the periplasmic space and properly processed to their mature sizes when expressed in *E. coli* (Seidler and Michel 1990, Meadows and Robinson 1991, Hashmol et al. 1994, Henry et al. 1997). Also, Mora and Cline (1998), and Wexler et al. (1998) reported that the signal

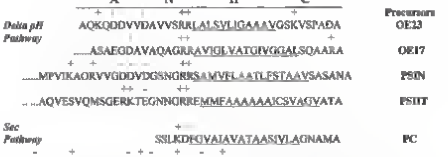


Figure 1-2. Lumen targeting domain (LTD) structure of precursors targeted to the cpSec, the Delta pH pathway, or to both pathways. The acidic (A) charged (N), hydrophobic (H) and cleavage (C) regions of LTD are shown. A hybrid dual targeting transit peptides (DT) is composed of the OE23 N region fused to the PC H region. The hydrophobic amino acids that comprise the H domain are underlined whereas charged amino acids are indicated with (+) or (-). The sequences of the LTD for OE23, OE33 and PC correspond to the mature terminus determined by Bassham et al (1991). Precursors: PC (plastocyanin from *Arabidopsis*), OE17, OE23, OE33 (17, 23 and 33-kD proteins of the oxygen-evolving complex from maize, pea and pea), PSIN from *Arabidopsis*; PSIIT from cotton, PSIF from spinach.

peptide from an *E. coli* precursor could direct thylakoid passenger protein into the thylakoids.

The general structures of LTDs for both cpSec and Delta pH pathways are the same. With a compatible passenger, N, H and C domains in the LTD are sufficient for precursor targeting (Henry et al. 1994, 1997). The function of the A domain is currently unclear. Certain sub-domains of LTD determinate pathway specificity. For the Delta pH pathway, the N domain contains the specificity element. All the Delta pH pathway precursors contain a double arginine (RR) motif in the N domain, which is essential for the Delta pH pathway transport (Chaddock et al. 1995, Henry et al. 1997). It is likely that a receptor would interact with the double arginines, possibly combined with the non specific hydrophobic core, and bring the precursor to the Delta pH pathway translocation machinery. In contrast to the Delta pH pathway, the H domain requirement is more specific for cpSec pathway precursor targeting, while the N domain requirement is non specific (Henry et al. 1997). So a different receptor could interact with the H domain and target the precursor to the cpSec pathway. The "domain determinant" hypothesis was further examined with artificial precursor DT-PC, which contains determinants for both pathways. DT-PC was constructed by replacing the N domain of cpSec pathway precursor PC with one from Delta pH pathway precursor OE23. This precursor incredibly can be efficiently transported on both pathways (Henry et al. 1997). Although "domain determinant elements" are key factors for pathway specificity, they alone seem insufficient for targeting. For example, amino acid residues surrounding the RR motif are important for the Delta pH pathway targeting (Bogsch et al. 1997).

In addition, results from Henry et al. (1994, 1997) and Clausmeyer et al. (1993) indicate that the mature protein plays a role in targeting and/or translocation on the cpSec pathway. For example, although a cpSec LTD could direct the Delta pH pathway passenger OE17 transport on the cpSec pathway, the transport was inefficient. Secondly, the hybrid (DT) transit peptide shown in Figure 1-2, while directing efficient transport of cpSec passengers on both pathways, was not able to direct efficient transport of the Delta pH passengers on the cpSec pathway.

In *E. coli* secretory proteins, a 30-residue-long region immediately downstream of the signal peptides has been termed the "export initiation domain". Introducing positively charged amino acid residues into the domain inhibits *E. coli* protein export at an early stage (Anderson & van Heijne 1991, 1993, Yoshihisa & Ito 1996). The inhibition could be reversed by adding negatively charged amino acids around the positively charged ones (Anderson & van Heijne 1991). Comparing the sequences of different precursors of the thylakoid cpSec pathway, we have observed that a 10-40 residue long region (adjacent to the LTD in PC and OE33, more distant from the LTD in PSII) is characterized as having net zero or negative charge and containing a pair of special amino acid residues (NN or NQ, N-asparagine, Q-glutamine). This region may be analogous to the "export initiation domain". I speculate that it is this region that may be involved in the interaction between the precursor mature sequence and cpSec pathway transport machinery. The Delta pH pathway precursors generally have a net positive charge in this region and lack NN or NQ. This may be the reason why the Delta pH pathway passengers cannot efficiently access the cpSec pathway.

Studies have shown that the Delta pH pathway may be capable of transporting tightly folded precursors (Clark et al. 1997; Hynds et al. 1998). The natural precursor OE23 of the Delta pH pathway appears to be in a tightly folded conformation prior to transport (Creighton et al. 1995). Transport on the cpSec pathway appears require an unfolded precursor conformation (Eoto et al. 1994, Hynds et al. 1998). So, it is also possible that Delta pH pathway precursor passengers cannot access the cpSec pathway because they have a tightly folded conformation.

Protein Transport on the cpSec Pathway

The belief that chloroplasts were originated from a prokaryotic endosymbiont has driven speculation that thylakoid transport would be homologous to protein export from contemporary bacteria. The Sec system for protein export in *E. coli* has been investigated in detail by both biochemical and genetic techniques (Pugsley 1993; Rapoport et al. 1996). A preprotein translocase has been identified in *E. coli* (Brundage et al. 1990). The translocase consists of an integral membrane protein complex SecY/E/G and a peripheral membrane protein SecA. A working model has been proposed by Hartl et al. (1990). In this model, chaperone SecB, by binding to the precursor in the cytosol, prevents the precursor from aggregation. SecA binds the precursor-SecB complex and brings the complex to SecY/E/G. ATP binds to SecA and its hydrolysis by SecA facilitates translocation of the precursor. Besides ATP, a proton-motive force is stimulatory for the Sec system operation.

Some evidence suggests that SecA may function as a primary receptor for the precursor in bacterial protein transport. The direct interaction of SecA with the secretory

proteins was demonstrated by means of chemical cross-linking with EDAC (1-Ethyl-3-[3-dimethlamnopropyl]-carbodiimide hydrochloride) and the interaction was signal peptide-dependent (Akita et al. 1990). A group of mutations in the SecA gene (termed prlD mutants) suppressed certain precursor *malE* signal peptide mutations. This suggested that SecA directly interact with the precursor signal sequence (Fikes and Bassford 1989, Puziss et al. 1989). SecA exists in both cytoplasm and membrane. It was suggested that cytosolic SecA does not function as the precursor receptor since, in reconstitution studies, an excess of SecA did not compete with SecY/E-bound SecA for limited amounts of added precursor-SecB complex (Hartl et al. 1990). Thus, it has been inferred that membrane association activates SecA for its receptor function. SecA is also known as the translocation ATPase of protein transport, so SecA could work as both an ATPase and a receptor.

There is also evidence to suggest that SecY is a precursor receptor. Antibodies against the N terminus of SecY are reported to prevent precursor binding to inverted membrane vesicles (Watanabe and Blobel 1989), as does trypsin treatment, which may inactivate SecY (Swidersky et al. 1990, 1992). Also, a group of mutations in the SecY gene (mutants prlA termed) suppress signal peptide mutations. By mapping the location of prlA mutations in SecY, Osborne and Silhavy (1993) hypothesized that the seventh transmembrane domain interacts with the hydrophobic core of the precursor signal peptide and plays a proofreading role. It is interesting that mutations in the SecE gene (prlG mutants) can also suppress signal peptides' mutations. So, it is possible that SecY or SecE may function as precursor receptors.

Thylakoid translocation of OE33 and PC has energy requirements similar to those of bacterial proteins that use the Sec system, implying that a Sec-like system is operational within chloroplast (Hulford et al. 1994, Yuan and Cline 1994a). Azide is known for its specific inhibition on SecA function in *E. coli* (Oliver et al. 1990). OE33 and PC transport is also inhibited by azide treatment, implying that a chloroplast homologue of SecA involved in the transport (Henry et al. 1994, Knott and Robinson 1994, Yuan and Cline 1994a). *SecA*- and *SecY*-homologous genes were found in the chloroplast genomes of several algae (Robinson and Kloseen 1994) and subsequently the homologous genes were identified in plants (Berghofer et al. 1995, Nohara *et al* 1995, Laidler et al. 1995). The homologous genes in plants are nuclear encoded and named *cpSecA* and *cpSecY*. The requirement of cpSecA protein for OE33 and PC transport was clearly demonstrated *in vitro* with purified cpSecA from pea chloroplasts (Yuan *et al* 1994). Purified cpSecA replaced stromal extract and reconstituted transport of OE33 and plastocyanin with buffer- or urea-washed thylakoids in an azide-sensitive manner. Hayard et al. (1997) observed a cpSecA-precursor crosslinking product during precursor binding. This at least indicates that the precursor is in direct contact with cpSecA. *In vivo* evidence for the cpSecA's role on the cpSec pathway protein transport came from the research with a maize mutant *tha1*: the maize *tha1* mutant was selectively inhibited in transport of OE33, plastocyanin and PSI-F (Voelker and Barkan 1995); the *Tha1* gene was isolated and shown to encode maize cpSecA (Voelker et al. 1997).

Protein Transport on the Delta pH Pathway

The Delta pH pathway was first revealed by the discovery that two lumen proteins, OE17 and OE23, were transported into the thylakoids in such a unique manner that no soluble factor or NTPs were required for the transport. The ΔpH across the thylakoid membrane was the sole energy requirement (Cline et al. 1992). Little is known about the operating mechanism for the Delta pH pathway. It appears to work like a typical export system in *E. coli* or ER. First, precursors using the system have classical signal peptides (Cline and Henry 1996). second, the precursor transport uses loop topology and is likely translocated into the lumen from amino terminus to the carboxyl terminus (Fincher et al. 1998. Fincher and Cline unpublished). Although the Delta pH pathway does not require any soluble components, it certainly requires proteinaceous components on the thylakoid membrane. Transport cannot be conducted with protease pre-treated thylakoids (Robinson et al. 1996). Also, transport is saturated with high level of precursor and competed by unlabeled precursor (Cline et al. 1993). A component named Hcf106 was identified as essential for Delta pH pathway function. Mutation in the *Hcf106* gene results in high-chlorophyll fluorescent (*hcf*) phenotype and causes accumulation of Delta pH pathway intermediate precursors in the stroma (Voelker and Barkan 1995). Although the mutation diminishes the ability of the thylakoids to generate and maintain a transmembrane pH gradient, the ΔpH present was as high as in *Tha1* which transports precursor at normal rate on the Delta pH pathway. Isolated chloroplasts from *hcf106* mutant seedlings were totally unable to transport OE17 on the Delta pH pathway but were still capable of transporting OE33 on the cpSec pathway (Settles et al. 1997). The *Hcf106* gene was cloned and the antibodies against Hcf106 localized the protein on

thylakoid membranes. The bulk of the Hcf106 protein is exposed to the stroma, suggesting that it may function as a receptor. Precursors on the Delta pH pathway have a critical RR motif in their lumen targeting domain. If Hcf106 functions as a receptor, it is likely that the RR is interacting with Hcf106 during the targeting process.

The Delta pH pathway was once considered to be eukaryotic innovation having evolved after endosymbiosis because all of its known substrates are absent from cyanobacteria, from which the chloroplast is believed to be evolved. However database searches with the maize Hcf106 sequence revealed a homologous class of hypothetical bacterial proteins. This evidence strongly suggested that a similar pathway is operating in bacteria. The existence of the system in bacteria was also predicted by Berks (1996) based on the observation that a class of periplasmic proteins in bacteria are synthesized with signal peptides containing an RR motif. These redox proteins are apparently exported together with their redox cofactors and the exported proteins may be in a folded conformation. *E. coli* contains three homologues of Hcf106 TatA, TatB and TatE, where tat stands for twin-arginine translocation. *tatA* and *tatB* genes are located in a four-gene operon, whereas the tatE is unlinked. *tatA* and *tatE* gene products have recently been shown by Sargent et al. (1998) to be required for the export of a range of proteins bearing the twin-arginine motif in their signal peptides. The Sec-system was shown to be unaffected in the mutant strains in which these genes were disrupted. Mutation of tatB also resulted in defective transport of RR-precursor (Weiner et al. 1998). Disruption of the *tatC* gene, which is also a member of the tat operon, results in a complete block in transport of five tested RR-precursor (Bogsch et al. 1998). TatC is a multispanning membrane protein and its homologous genes are present in a range of bacteria, plastids,

and mitochondria. Thus the Delta pH pathway is a novel pathway, discovered in plant thylakoids, but apparently exists in prokaryotes.

Summary and Perspective

Protein transport is required for the biogenesis of the chloroplast, the site of photosynthesis. Hundreds of chloroplast proteins are encoded in the cell nucleus and are synthesized in the cytosol. These proteins have to be transported into the chloroplast and functionally assembled.

A nuclear-encoded chloroplast protein is initially synthesized as a precursor protein that contains a transient transit peptide at its amino terminus. For a thylakoid lumen protein, its transit peptide consists of two parts: one is a stroma-targeting domain (STD) and another is a lumen-targeting domain (LTD).

Import of the precursor across the chloroplast envelope is governed by the STD of the transit peptide and accomplished by the cooperation of machinery components on both outer and inner envelope membranes. Such machinery is collectively called the general import apparatus. ATP hydrolysis is used to power import. The STD is cleaved by a processing protease in the stroma, resulting in either a mature stromal protein or an intermediate thylakoid protein precursor which is further directed to the thylakoids by the exposed LTD.

There appears to be one major pathway for protein import across the chloroplast envelope. However, there are two pathways for protein transport into the thylakoid lumen. One pathway is called the cpSec pathway since this pathway employs a chloroplast homologue of the bacterial SecA protein. A second pathway is named the

Delta pH pathway because in this pathway, the ΔpH across the thylakoid membrane is the only energy requirement for protein transport. Each pathway is specific for a subset of precursor proteins.

Protein transport into the thylakoid lumen is directed by the LTD. All precursors on the cpSec pathway or Delta pH pathway have a similar basic LTD structure containing three essential domains: N, H and C. Targeting to the cpSec pathway requires a specific hydrophobic H domain. Targeting to the Delta pH pathway requires a specific N domain (Henry *et al* 1997). A critical RR motif in the N domain is required for committing the precursor to the Delta pH pathway (Chaddock et al. 1995). The RR motif is thought to interact with a putative receptor on the thylakoid membrane. Further information is necessary to establish how the LTD is interacting with transport machinery and whether the precursor mature part is involved in the targeting step.

The cpSec pathway is powered by ATP hydrolysis and stimulated by ΔpH (Cline et al. 1992). The soluble component cpSecA is required for the transport (Yuan et al. 1994). Chloroplast homologues of bacterial SecY have been cloned (Laidler et al. 1995) and recent studies show that cpSecY is functional on the cpSec pathway (Hiroki et al. in preparation). Although Howard et al. (1997) demonstrated that precursor can form a complex with cpSecA on the thylakoid membrane, it was not clear from that study whether the cpSecA functions as a receptor or even if the interaction between precursor and cpSecA is specific. Also it is not known whether the interaction involves cpSecY. Further work is required to show how cpSecA engages precursors in targeting and translocation.

The ΔpH is the only energy requirement for the Delta pH pathway transport and no soluble component is required (Cline et al. 1992). The thylakoid protein Hcf106 was recently identified as a component required for the pathway (Settles et al. 1997). Although the Delta pathway was first discovered by a biochemical approach, the machinery components were identified by genetic methods. However, development of new biochemical approaches is necessary to delineate the role of identified components in the translocation mechanism.

In the studies presented in this dissertation, I have conceptually and experimentally divided protein transport across the thylakoid membrane into two steps. The first is targeting in which the precursor is committed to the pathway. The second is translocation, wherein the protein actually is moved by the transport machinery across the membrane. I hypothesize that targeting results from a specific interaction between the precursor and components of the protein transport machinery. Each protein transport pathway has its own components to recognize the precursor and such components are referred as precursor *receptors*. So far, protein transport machinery components (receptors) related to pathway selection have not been identified.

The purpose of this study is to examine the molecular determinants and steps of the process that result in precursor commitment on the cpSec pathway or the Delta pH pathway. The specific questions addressed are: 1. Is there an earliest detectable precursor targeting step that commits the precursor on the cpSec pathway or the Delta pH pathway? Specifically, is there a detectable complex formed during the precursor targeting step? 2. Are there any receptors that interact specifically with the precursor during the precursor

targeting step? 3 What are the elements of precursors necessary for targeting? 4 What are the energy requirements for precursor targeting and translocation?

The long range goal is to determine underlying reasons for the existence of multiple pathways for protein transport into the thylakoids. To achieve the goal, it will first be necessary to define each pathway The successful completion of this study will provide further understanding of thylakoid protein transport mechanisms

CHAPTER 2
PROTEIN TARGETING AND TRANSLOCATION ON THE cpSEC PATHWAY

Abstract

To investigate the basis for pathway specific targeting, the characteristics of membrane binding and chase of precursors on the cpSec pathway were examined. Previous studies showed that the cpSec pathway precursor tOE33 could form a productive intermediate and be crosslinked to cpSecA on the thylakoid membrane (Haward et al. 1997). Our results support the notion that precursor commitment to the thylakoid cpSec pathway occurs on the membrane, rather than in the soluble stroma. Precursor binding to the thylakoid membrane required stromal protein, of which cpSecA was the essential component, and occurred when transport was inhibited by removing ATP from the assay with apyrase. Sodium azide and nonsphares, although inhibiting transport, did not result in increased precursor binding. Subsequent transport of bound precursor required ATP, but was not stimulated by additional cpSecA. Upon interaction with thylakoids, precursors could be crosslinked into a high molecular weight complex (cpSec complex) that migrated anomalously in SDS-PAGE. The crosslinked product was immunoprecipitated with antibodies to cpSecA as well as with antibodies to cpSecY. Formation of the cpSec complex was strictly precursor-specific. Precursors capable of being transported by the cpSec pathway were crosslinked into the complex, whereas precursors transported by the Delta pH pathway and SRP pathway were not. Of interest is the fact that precursors consisting of a cpSec-compatible signal peptide fused to a Delta

pH passenger protein produced a higher molecular weight crosslinking product that was not immunoprecipitated by antibodies to cpSecA. Previous studies have shown that Delta pH passenger proteins are incapable of efficient transport on the cpSec pathway. This later result suggests that commitment to the cpSec pathway at an early step involves not only the signal peptide, but elements of the passenger protein as well.

Introduction

Nuclear-encoded thylakoid lumen-resident proteins are synthesized in the cytosol as precursors with bipartite amino-terminal transit peptides. Precursors are localized to the lumen by a two-step mechanism (Cline and Henry 1996, Schnell 1998, Robinson et al. 1998). In the first step, the precursors are imported across the chloroplast envelope into the stroma. This step is governed by the stromal targeting domain of the transit peptide. A stromal processing protease removes the stromal targeting domain, thereby producing a stromal intermediate precursor, which is transported across the thylakoid membrane in the second step. Thylakoid transport is directed by the lumen-targeting domain of the transit peptide, which is removed by the thylakoid processing protease.

The cpSec pathway is one of two pathways for protein transport into thylakoid lumen. OE33, PC and PSIF (F sub-unit of Photosystem I) are three proteins known to use the cpSec pathway for their transport. Protein transport on the cpSec pathway requires ATP, a stromal protein cpSecA (Yuan et al. 1994) and a membrane protein cpSecY (Mori et al., in preparation). According to Hayard et al. (1997), when ATP was eliminated with apyrase, rOE33 transport was inhibited, resulting in accumulation of bound precursor on the thylakoid membrane. The bound precursor could be chased into

thylakoid lumen upon ATP addition. Precursor binding is considered to be a targeting step whereas the chase is the translocation step. cpSecA and cpSecY are homologues of the bacterial SecA and SecY proteins (Berghofer et al. 1995, Nohara et al. 1995, Laidler et al. 1995). The bacterial SecA protein is a translocation ATPase that binds to precursors and SecY and "pushes" peptide segments across the membrane through a cycle of insertion and deinsertion. SecY is a membrane protein that is thought to play a major role in forming the protein translocation channel with SecE component (Akiyama and Ito 1985, Meyer et al. 1999).

Committing a precursor protein to a specific pathway, i.e. targeting, is believed to be initiated by an interaction between precursor and a receptor. It has been shown that the lumen-targeting domain determines whether a protein is transported on the cpSec or Delta pH pathway (Henry et al. 1994). It has also been shown that the mature protein domain, i.e. the passenger, can greatly affect the efficiency of transport by the cpSec pathway. For example, although a cpSec LTD directed transport of OE17 on the cpSec pathway, the transport was inefficient. Secondly, the hybrid (DT) transit peptide, while directing efficient transport of Sec passengers on both pathways, was only able to direct efficient transport of Delta pH passengers on the Delta pH pathway (Henry et al. 1994, 1997). Haward et al. (1997) reported that tOE33 formed a complex on the thylakoid membrane with cpSecA and suggested that cpSecA may function as a receptor. However, it was not known which part of the precursor bound to cpSecA and more information is required to determine whether cpSecA functions as a receptor.

I am interested in learning how the cpSec pathway system recognizes its respective substrates and commits them to the pathway. I have attempted to describe and

characterize the first committed step on the cpSec pathway and to define in general terms the sequences required for commitment. My results show that the first observable interaction of precursors with the cpSec pathway occurs on the membrane and results in formation of a large complex that includes precursor, cpSecA, and cpSecY. This interaction requires a cpSec LTD and additionally a cpSec-compatible flanking region. Proteins that possess dual targeting signal peptides fused to Delta pH passenger proteins are capable of binding to the membranes as well as chasing into the lumen on the delta pH pathway. However, these proteins do not form a complex with cpSecA.

Materials and Methods

Preparation of precursors

In vitro transcription plasmids for tOE33, tOE23 and LHCP were described as Cline et al. (1993). Preparation of tPC, DT33, DT17 and DT23 were described as Henry et al. (1997).

Preparation of Chloroplasts, Lysates, Thylakoids and Stroma

Chloroplasts were isolated from 9 to 10-day-old seedlings of pea (Laxton's Progress 9). Lysates, thylakoids and stromal extract were prepared from the intact chloroplasts (Cline et al. 1993).

Preparation of Purified cpSecA

CpSecA was purified from stromal extract as described by Yuan et al. (1994b), except that studies reported here used cpSecA obtained after the Mono-Q ion exchange step. The concentration of purified cpSecA was estimated by Coomassie staining of SDS polyacrylamide gels using BSA as a standard.

Thylakoid Protein Transport Assay

Transport assays were conducted essentially as described previously (Cline et al. 1993) 25 µg chlorophyll of thylakoids or lysates in 25 µl of import buffer (50 mM Hepes buffer pH 8, 0.33 M sorbitol) was mixed with 30 µl HKM buffer (10 mM Hepes buffer pH 8, 10 mM $MgCl_2$) with or without 300 µg of stromal protein, and incubated with 20 µl 1 fold diluted *in vitro* translated precursor in import buffer with 30 mM leucine. ATP or apyrase, nnophores, or azide were added in respective assays The total volume of each assay was about 80 µl. Assays were incubated under 70 µE $m^{-2}s^{-1}$ white light (or dark) for 15-30 minutes at 25°C. After incubation, thylakoids were recovered by centrifugation and were washed once with import buffer One half of each sample of recovered membranes was analyzed directly on SDS-PAGE The other half was treated with 10 µg thermolysin in 100 µl of import buffer (Cline 1986) and then analyzed by SDS-PAGE and fluorography

Precursor Binding and Chase Assays

Thylakoids or chloroplast lysate (in 25 µl import buffer) equivalent to 25 µg chlorophyll was mixed with 40 µl HKM buffer with or without 400 µg of stromal protein. Two µg cpSecA, nnophores, or azide were added to respective assays. The mixture was incubated for 10 minutes in the dark in the presence of 2 µl (units) apyrase. About 10 µl *in vitro* translated precursor was added and the incubation continued for 15 minute at 25°C in the dark. Following the incubation, thylakoid membranes were recovered by centrifugation, washed twice with import buffer and divided into two equal portions in fresh microfuge tubes. One portion was analyzed directly. The other portion was incubated under chase conditions. The chase was conducted under 70 µE $m^{-2}s^{-1}$ white

light for 15-30 minutes at 25°C in a total of 37.5 µl of import buffer containing ~200 µg protein of stromal extract and 1mM DTT. Where designated, 12.5 µg chlorophyll aliquots of thylakoids recovered from binding or chase samples were treated with 10 µg thermolysin in 100 µl of import buffer (Cline 1986).

Preparation of Urea washed or Thermolysin Treated Thylakoids for Binding Assays and Urea or Na_2CO_3 Wash of the Thylakoid Bound Precursor

Urea-washed thylakoids were prepared by incubating 0.5 mg chlorophyll of thylakoids in 1 ml of 2 M urea in import buffer for 8-10 minutes on ice. Thylakoids were recovered by centrifugation and washed once with import buffer. Thermolysin-treated thylakoids were prepared by incubating 0.33 mg chlorophyll of thylakoids in 1 ml import buffer containing 100 µg thermolysin on ice for 40 minutes. Thylakoids were recovered by centrifugation and washed twice with import buffer containing 10 mM EDTA and once with import buffer. Urea or Na_2CO_3 wash of the thylakoids recovered from binding assays was accomplished by incubating 0.5 mg chlorophyll/ml of thylakoids with 6 M urea or 0.1 M Na_2CO_3 (in water) on ice for 30 minutes. The thylakoids were recovered by centrifugation (at 3200x G for 8 minutes) and washed twice with import buffer.

Cross-linking Reactions

Binding assay mixtures, following incubation for 15 minute, received 0-2 mM cross-linker from 0-40mM stocks in DMSO (for DSS (disuccinimdyl suberate), MBS (m-Maleimidobenzoyl-N-hydroxysuccinimide ester) and APDP (N-[4-(p-Azidosalicylamido) butyl]-3'-[2'-pyridyldithio] propionamide)) or H_2O (for BS^3 (bis [sulfosuccinimidyl] suberate)). Cross-linking was allowed to proceed at room temperature for 30 min or at 0°C for 2 hours and was terminated with 50 mM Tris-HCl

pH 7.5 from a 1 M stock. After the cross-linking reaction, the membrane fraction was recovered by centrifugation and washed with import buffer.

Preparation of Antibodies

LHCP and CpSecA antibodies were prepared as described by Payan and Cline (1991) and Yuan et al. (1994b) respectively. Peptides NH_2-CKLQDLQKKEGEAGRKK-COOH and NH_2-CDDVSEQLKRQGASIPLVRPGK-COOH, named In1 and In2 respectively, correspond to two internal stromal-facing regions of cpSecY. In1, In2 and the antibodies against the peptides were prepared by Bio-Synthesis company (TX). In1 and In2 were conjugated via their N-terminals to carrier keyhole limpet hemocyanin with crosslinker MBS and the crosslinked products were used as antigens. The peptide NH_2-CRAEHSQKYNIELYDFDKY-COOH (named C-term), corresponding to the C-terminal stromal-facing region of pea cpSecY, was made and cross-linked by Genosys Biotechnologies company (Woodlads, TX). The antibodies raised against the Keyhole limpet hemocyanin-linked C-term peptide were prepared by Cocalico Company and described by Mori et al. (In preparation).

Immunoprecipitation of the Cross-linked Complex

Twenty-five μg chlorophyll of thylakoid membrane recovered from each cross-linking assay was resuspended in 75 μl of 10 mM HEPES/KOH (pH 8), 10 mM $MgCl_2$, followed by 4 μl 20% SDS. After 30 minute incubation at 37°C, the sample was brought to 800 μl with 10 mM HEPES/KOH (pH 8), 10 mM $MgCl_2$, 0.2% Triton X-100. Ten to fifteen μl serum, with or without 10-20 μg synthetic peptides, was added and the sample was mixed end-over-end at 4°C overnight. Forty μl 1:1 protein A Sepharose slurry in 10 mM HEPES/KOH (pH 8), 10 mM $MgCl_2$ was added and the sample was mixed end-

over-end at 4°C for 1 hour. The Sepharose beads were pelleted by centrifugation at 500x G for 3 minutes, resuspended in 10 mM HEPES/KOH (pH 8), 10 mM $MgCl_2$, transferred to a new tube, pelleted, and then treated with 20 µl 2X SDS sample buffer.

Analysis of Samples

Samples recovered from the assays were subjected to SDS-PAGE and fluorography. Most samples were analyzed with 12.5% acrylamide concentration gels. Cross-linking and immunoprecipitation samples were analyzed with 7.5% gels. Quantification of the amount of radiolabeled proteins resulting from these assays was accomplished by scintillation counting of radiolabeled proteins extracted from excised gel bands (Cline 1986).

Results

No Interaction can be Detected between cpSec Pathway Precursor and Soluble cpSecA

CpSecA is the only soluble chloroplast component required for protein transport on the cpSec pathway and, therefore, the only candidate for a specific interaction in the soluble fraction. Bacterial SecA is reported to interact weakly with precursor in solution (Hartl et al. 1990) and can be crosslinked to precursors with EDAC (1-Ethyl-3-[3-dimethlaminopropyl]-carbodiimide hydrochloride) (Akita et al. 1990). In preliminary studies, we were unable to detect any interactions between cpSec pathway precursors and cpSecA in solution using several different interaction assays. These included non-denaturing PAGE (gel shift assays), gel filtration of the authentic stromal intermediate

isolated from chloroplasts, crosslinking combined with immunoprecipitation, and nascent chain crosslinking combined with immunoprecipitation (data not shown).

tOE33 Binds to the Thylakoid Membrane when Transport is Prevented by Eliminating the ATP

In *E. coli*, strong binding to Sec components occurs on the membrane (Hartl et al. 1990). In addition, Haward et al. (1997) reported that the precursor tOE33 binds productively to thylakoids and can be crosslinked to cpSecA. Here we have further characterized this binding and investigated the possibility that membrane binding represents the first committed step on the cpSec pathway. [^{3}H]-labeled precursor was incubated with thylakoids under assay conditions either conducive for transport or where a single transport requirement was withheld (Figure 2-1). Wheat tOE33 was used as the precursor as it is the most efficient cpSec pathway substrate under our conditions. As in previous studies (Yuan et al. 1994a, Hulford et al. 1994), tOE33 was transported into the thylakoid lumen when stromal extract, ATP and ΔpH were present, as judged by processing to the mature size and inaccessibility to exogenous protease. Transport was inhibited when assays were conducted in the presence of ionophores that dissipate ΔpH, or azide, a SecA inhibitor, or in the absence of ATP (apyrase was used to scavenge all traces of ATP). In the absence of ATP/presence of apyrase, precursor binding to the thylakoids membrane was stimulated (Figure 2-1, *lane 1* and *lane 4*). The amount of bound precursor was ~ 2.6 fold higher in the presence of apyrase than that under any of the other conditions. In the presence of apyrase, about 2.6% of the added precursor in the transport mixture was bound on the thylakoid membrane. Of interest is that, although

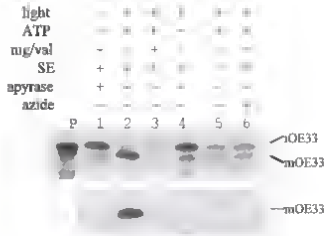


Figure 2-1 cpSec-pathway transport intermediate accumulated on the thylakoid membrane when transport was arrested with apyrase Transport assays with [^{3}H]-leucine labeled tOE33 precursor were conducted with isolated thylakoids under conditions (shown above the panel) designed to test the effects of removing ATP or stromal extract (SE), of dissipating the ΔpH, or of inhibiting cpSecA with azide Assays (75μl) contained 412.5 μg protein of stromal extract (SE), 5 mM Mg-ATP, nigericin and valinomycin (nig/val) at 0.5 μM and 1 μM, respectively, and 10 mM sodium azide as indicated above the panel. Apyrase treatment was 2 unit per 75 μl assay For the top panel, the thylakoid membranes were recovered by centrifugation and washed with import buffer Membranes in the lower panel were treated with the protease thermolysin prior to washing Samples were then analyzed with SDS-PAGE and fluorography Each lane contained 12.5 μg chlorophyll (100%) of thylakoid membranes present in each assay *Lane P* represents 0 25 μl (5%) precursor added to the assay reaction. The positions of the precursor tOE33 and the mature form (mOE33) are marked.

ionophores and azide inhibited transport of iOE33, they did not result in increased binding.

Binding of iOE33 to Thylakoids is Productive and Stimulated by cpSecA

To determine if precursor was productively bound in the absence of ATP/presence of apyrase membranes, washed following a binding assay, were incubated with stromal extract and ATP at 25°C for 30 minutes (Figure 2-2). Approximately 11% of the bound precursor was subsequently transported (chased) into the lumen and processed to mature size, whereas precursor bound without stromal extract or cpSecA failed to be chased into the lumen (Figure 2-2*A*). cpSecA is the required stromal component for iOE33 transport (Yuan et al. 1994b). Purified cpSecA significantly increased precursor binding and the subsequent chase over that of buffer alone. The amount of cpSecA added is approximately equivalent to the amount in unfractionated stromal extract. The relatively low percentage of precursor that is chased under our conditions may reflect the fact that transport competence of thylakoids is significantly reduced by the washing steps. This is shown in Figure 2-2*B*. A transport assay conducted with apyrase treated and buffer washed thylakoids plus stromal extract yielded ~56% transport compared to freshly prepared membranes (compare *lane 1* to *lane 4*).

It was shown in Figure 2-1 that azide or ionophores alone, while inhibiting transport, did not result in increased precursor binding. Figure 2-3 examines the effects of azide and the ΔpH on binding in the absence of ATP/presence of apyrase. Azide slightly decreased, whereas ionophores had no significant effect on binding. Precursor bound under all those conditions was chased into the lumen, although with varying efficiencies. Of interest is

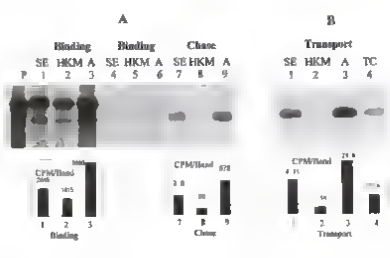


Figure 2:2 cpSecA stimulates tOE33 binding to the thylakoid membrane and the binding is productive. *Panel A* 15 µl [^{3}H]-labeled tOE33 precursor *in vitro* translation product was incubated with 37.5 µg chlorophyll *a/b* thylakoids (in 37.5 µl import buffer) in the presence of 3 µl (equal to 3 units) apyrase in import buffer for 15 minute at 25°C in a total 115.5 µl binding assay. Binding assays received ~800 µg of stromal protein in 60 µl 10 mM Hepes/KOH pH 8, 10 mM $MgCl_2$ (HKM buffer) (*lanes 1–4–7*), or 60 µl HKM alone (*lanes 2–5–8*), or ~3µg purified cpSecA in 60 µl HKM buffer (*lanes 3–6–9*). After binding incubation, the thylakoid membranes were recovered, washed twice with import buffer, divided into three equal aliquots and transferred to new tubes. One aliquot was directly analyzed (*lanes 1–3*). A second aliquot was treated with thermolysin (*lanes 4–6*). The third aliquot was incubated under chase conditions and then treated with thermolysin (*lane 7–9*). Each lane contains 100% of thylakoid membrane from each assay. *Lane p* represents about 0.25 µl precursor *in vitro* translation product. *Panel B*. Thylakoid transport assays conducted with fresh thylakoids (*Lanes 1–2–3*) or thylakoids incubated with apyrase under conditions of a binding assay and then washed twice with import buffer before use (*lane 4*). All reactions contained 5 mM ATP. Reactions of *lanes 1–4* contain stromal extract, *lane 2* buffer, *lane 3* purified cpSecA. Final samples were treated with thermolysin before being analyzed by SDS-PAGE and fluorography. The charts display radioactivity (CPM) of corresponding bands.

Figure 2-3 The effects of ΔpH and azide to rOE33 binding to the thylakoid membrane rOE33 binding assays were conducted in the presence of stroma and apyrase Binding assays in lane 2, 3 and 4 were supplemented with 3 μM CCCP (Carbonylcyanide 3-chlorophenylhydrazone), 0 5 μM/1 μM nig/val or 10 mM sodium azide respectively Chase assays were all conducted under identical condition (see Methods)

that precursor bound in the presence of ionophores was chased into the lumen with efficiency greater than precursor bound under other conditions. Because ionophore effects of nig/val persist through washing steps, that was the first indication that a ΔpH was not required for the chase reaction.

Soluble cpSecA is not Required for the Chase of Bound Precursor

The requirements for chase of bound precursor into the thylakoid lumen were investigated (Figure 2-4). Maximum chase of bound 1OE33 resulted when stromal extract was included in the re-incubation mixture. ATP alone was able to partially stimulate chase, but chase with ATP plus stromal extract was no greater than stromal extract alone. Purified cpSecA was ineffective in stimulating chase, indicating that cpSecA is committed at the early binding step and that free cpSecA is largely ineffective at the chase stage. This is consistent with observations in the bacterial Sec system that excess soluble SecA did not compete with membrane-bound SecA for limited amounts of added precursor-SecB complex (Hartl et al. 1990) and that bacterial SecA does not cycle off the membrane during protein transport (Cheo et al. 1996). Although free cpSecA was not required for the chase, bound cpSecA played an important role during the chase since azide partially inhibited the chase (Figure 2-4*B*). Unexpectedly, the chase was stimulated by ionophores. Yet, the thylakoids' ability to generate a ΔpH was not likely destroyed by manipulations involved in binding and washing because apyrase treated and washed thylakoids were still capable of transporting fresh precursor in an ionophore-sensitive fashion (data not shown). Rather, the energy required for transport was derived from ATP because addition of apyrase prevented chase of precursor into the lumen (data not

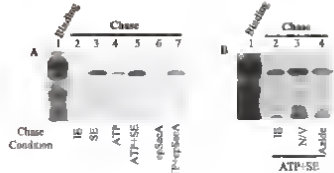


Figure 2-4 Requirements for chase of bound precursor into the lumen. A. 28 µl [^{3}H]-leucine labeled 1OE33 precursor was incubated with 87.5 µg chlorophyll of chloroplast lysate (in 110 µl import buffer) and ~1200 µg protein of stromal extract (in 120 µl HKM buffer) in the presence of 7 units (µl) apyrase for 15 minute at 25°C. Thylakoid membranes were then recovered and washed twice with import buffer and divided into seven equal portions. Each portion was transferred to a new tube. One portion was analyzed directly (*lane 1*). The remaining portions were further assayed for chase under different conditions. Assays contained import buffer (*lanes 2–4*), 200 µg stromal protein (SE)(*lanes 3–5*), ~0.8 µg purified cpSecA (*lanes 6–7*) were incubated in the presence (*lanes 4–5–7*) or absence (*lanes 2–3–6*) of 5 mM ATP for 30 min. at 25°C in light in a total 37.5 µl. Recovered thylakoids were treated with the thermolysin and then analyzed by SDS-PAGE and fluorography. B. The binding incubation was similar to that shown in *panel A*. During the chase step, 12.5 µg chlorophyll of thylakoid membrane with bound precursor were resuspend in buffer containing 200µg protein of stroma extract and 5 mM ATP plus 1 µM/0.5 µM nigericin/valinomycin (N/V) or 10 mM sodium azide (Azide) or import buffer (IB).

shown). Stromal extract contains small amounts of ATP and presumably this was sufficient to power the transport reaction in assays containing stromal extract alone

Precursor Binding to Thylakoids is Mediated by Protein-protein Interactions

Further investigation into the nature of the observed precursor binding is shown in Figure 2-5. Binding was maximal with membranes that were either buffer- or urea-washed and when stromal extract was present in the reaction mixture. Approximately 2.1-2.7 % of the added precursor was bound to the membrane under these conditions. Binding to protease-treated membranes was greatly reduced (only ~0.4% of the added precursor was bound to the membrane). Following the binding reaction, thylakoids were washed with 0.1 M Na_2CO_3 or 6 M urea to assess the nature and strength of the binding. The majority of precursor bound to buffer- or urea- prewashed membranes (~60%) was removed by the Na_2CO_3 and urea washes, whereas the low level of precursor bound to protease-treated membranes was largely resistant. Thus, combined with previous observations that transport is eliminated by protease (Robinson et al. 1996), but not urea-treatment of thylakoids (unpublished data) suggests that rOE33 binding involves protein-protein interaction, whereas a low level of precursor can non-specifically interact with the thylakoid surface.

Productive Binding Results in Formation of a Complex on Thylakoids

The above results imply that binding of rOE33 involves cpSecA and thylakoid protein(s), and suggests the formation of a complex on the membrane, similar to the Sec complex shown for bacterial membranes (Brundage et al. 1990). To assess whether such a complex exists, a binding reaction was conducted for 15 minutes, after which the cross-

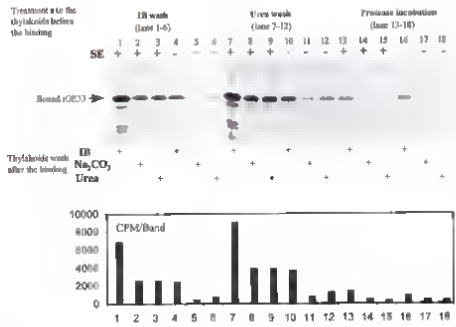

Treatment s to the thylakoids before the binding
IB wash (lane 1-6)
Urea wash (lane 7-12)
Protease incubation (lane 13-18)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
SE + + + - - - + + + - - - + + - -
Bound rOE33
Thylakoids wash after the binding
IB
Na_2CO_3
Urea
10000
8000
6000
4000
2000
0
CPM/Band
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 2-5 rOE33 precursor binding to the thylakoid membrane is mediated by protein-protein interactions. Nine µl [^{3}H] labeled rOE33 translation product was incubated with 37.5 µg chlorophyll of import buffer-washed thylakoids or 2 M urea-washed thylakoids or thermolysin (100 µg/ml)- treated thylakoids in the presence or absence of ~525 µg stromal proteins in a total 112.5 µl. All assays contained 3 unit of apyrase and were incubated in the dark at room temperature for ~30 min. After the incubation, each sample was divided into three equal portions. The thylakoid membranes in each portion were washed with import buffer (*lanes 1, 4, 7, 10, 13, 16*) or 0.1 M sodium carbonate (*lanes 2, 5, 8, 11, 14, 17*) or 6 M urea (*lanes 3, 6, 9, 12, 15, 18*). Thylakoid membranes recovered from the assays (12.5 µg chlorophyll) by centrifugation were analyzed by SDS-PAGE and fluorography

linking agent DSS (disuccinimidyl suberate) was added to stabilize any possible complex. Multiple cross-linking products, apparent as higher molecular weight radioactive bands, were present in both the soluble, stromal fraction and the thylakoid membrane fraction (Figure 2-6 *A, (1)* and *(2)*). Because of the requirement for cpSecA in the binding reaction, crosslinked products were subjected to immunoprecipitation with antibodies to cpSecA. Soluble crosslinking products were not immunoprecipitated (Figure 2-6*B*). However, the large crosslinking product that migrates near the top of the separating gel was immunoprecipitated from the membrane fraction with antibodies to cpSecA (Figure 2-6*A, (3)*) but not with preimmune serum (data not shown, but see Figure 2-7). Further experiments (Figure 2-6*B*) indicated that the formation of this crosslinked product required both stromal extract and the thylakoid membrane and that purified cpSecA could replace stromal extract in this reaction. From these data, it can be concluded that tOE33 forms a complex with other proteins on the thylakoid membrane and that cpSecA is part of the complex.

In a standard binding assay containing thylakoids and stromal extract, ~2.4% of the added precursor remained bound to the recovered and washed thylakoid membranes. When 1 mM DSS was added at the end of the binding reaction, ~3.5% of the added precursor was recovered as the cross-linked complex. Over 50% of the cross-linked complex was immunoprecipitated with antibody against cpSecA. When thylakoids recovered from a binding assay were treated with DSS following the washing steps, the same cross-linked complex was formed, though a lesser amount than when crosslinker was added directly to the binding reaction (data not shown). When the binding assays were conducted in the presence of ionophores or sodium azide alone, cpSecA-containing

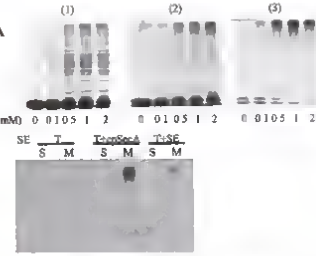


Figure 2-6. Formation of a complex between precursor and components of the cpSec machinery. A. Crosslinkers stabilize a large cpSecA-containing complex on the membrane. Ten µl [^{3}H]-labeled iOE33 *in vitro* translation product was incubated with 25 µg chlorophyll of chloroplast lysate plus 40 µl (400 µg of protein) additional stromal extract in the presence of 2 units (in 1µl import buffer) apyrase. After 15 minutes incubation, 4 µl of varying amounts of DSS in DMSO were added to the reaction mixture, which was further incubated for 2 hours in an ice bath. The final DSS concentrations in the mixture were 0-2 mM. The cross-linking reaction was terminated by adding 4 µl 1 M Tris-HCl, pH 7.5. The membrane and stromal fractions were separated by centrifugation and aliquots of the samples were further analyzed by anti-cpSecA immunoprecipitation. Final samples were analyzed by SDS-PAGE (7.5% gels) and fluorography. *Panels: (1)*, the cross-linking products in 7.5 µl supernatant from cross-linking mixture. *(2)*, the cross-linking products on the 6.25 µg chlorophyll of membrane. *(3)*: the antiSecA immunoprecipitation products of the cross-linking products on the about 12.5 µg chlorophyll of membrane. B. cpSecA is required for complex formation. Ten µl [^{3}H]-labeled iOE33 precursor was incubated either with stromal extract (SE, ~600 µg protein) or with 25 µg chlorophyll of thylakoids (T) or with the thylakoids plus purified cpSecA (T+SecA, 1.6 µg cpSecA) or with the thylakoids plus stroma extract (T+SE) and the reactions were crosslinked with 1 mM DSS. The reactions were then subjected to immunoprecipitation with antibody to cpSecA. Immunoprecipitation products from the soluble fraction (S) or the membrane (M) are shown.

crosslinking products were not obtained (data not shown), indicating a strong correlation between productive binding and the ability to form a crosslinked complex.

cpSecY is Part of the cpSec Complex

In bacteria, the interaction of precursor and SecA with the membrane results in a complex with the SecY/E/G protein. To determine if the precurser-cpSecA complex also contains cpSecY, immunoprecipitations were conducted with antibodies generated to cpSecY. Antibodies were raised to synthetic peptides that correspond to several regions of the predicted pea cpSecY sequence (see Methods). Initially, antibodies were raised to peptides corresponding to two internal stromal-facing regions of cpSecY. These antibodies are capable of specifically immunoprecipitating the cpSecY translation product but fail to give a band on immunoblots (Mori and Cline, unpublished). Subsequently, a third antibody was raised to the extreme carboxyl terminus of cpSecY. This antibody immunodecorates a 42 kD pea thylakoid protein, presumed the cpSecY, located in the stroma lamellae (Mori et al., in preparation). All three antibodies immunoprecipitated the large precursor cpSecA complex (Figure 2-7 A and B). The complex was not precipitated with pre-immune serum or when immunoprecipitation was conducted in the presence of the corresponding synthetic peptides. To verify that DSS cross-linker was not simply crosslinking the bound precursor to any component on the thylakoid membrane, immunoprecipitations were conducted with antibodies against LHCP, a major protein of the thylakoid membrane but not related to the cpSec pathway. No radiolabeled cross-linking products were immunoprecipitated with these antibodies (Figure 2-7C).

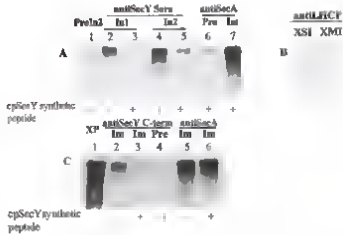


Figure 2-7 cpSecY is part of the cpSec complex. The experimental conditions were similar to Figure 2-6. Cross-linking reactions were conducted with 1 mM DSS. A. Cross-linking products on 25 µg chlorophyll of thylakoid membranes were immunoprecipitated with antibodies against two internal stroma-facing regions of cpSecY (In1, In2 synthetic peptides) in the presence or absence of the corresponded synthetic peptides (1-2ug/ul serum). AntiSecA immunoprecipitation in the presence of SecY-synthetic-peptide (In2) was used as control. The preimmune sera for both antiepSecY (anti-In2) and antiSecA (*Lane 1* and *Lane 2* respectively) were also used as controls. B. tOE33 precursor cross-linking products from both the stroma (XSI) and the thylakoid membrane(XMI) were immunoprecipitated with antiLHCP serum. C. Cross-linking products on the 25 µg chlorophyll of thylakoid membrane were immunoprecipitated with antibodies against the extreme carboxyl terminus of cpSecY in the presence or absence of the cpSecY synthetic peptide. Anti cpSecA immunoprecipitations in the presence or absence of cpSecY synthetic peptide demonstrated the specificity of the peptide effect. The preimmune sera of cpSecY antibody (Pre) was also used as control. *Lane 1* contains cross-linking products of 12.5 µg chlorophyll of thylakoid membrane. The remaining lanes contain immunoprecipitate obtained from 25 µg chlorophyll of crosslinked thylakoids.

Since the cpSec complex contained cpSecY, we further wanted to know if cpSecY played any role in the precursor binding. Antibodies against the C-terminal region of cpSecY inhibited protein transport on the cpSec pathway (Mori et al., in preparation). After thylakoids were pre-incubated with these antibodies, precursor binding on the thylakoid membranes were significantly reduced. The corresponding antigen reversed the precursor binding reduction (Figure 2-8).

Formation of the cpSecA-containing Complex is Pathway-specific

It has been shown that precursor proteins transported by the Delta pH pathway and cpSRP pathways do not require cpSecA (Yuan et al. 1994). Therefore these precursors are not expected to form a cpSecA containing complex. Similar crosslinking reactions were conducted with precursors from different pathways (Figure 2-9, *Panel A*). Several different crosslinking agents were used to ensure that a crosslinking product would be obtained if precursors were in contact with cpSecA. CpSecA-containing complexes were obtained with tOE33 and tPC, both are substrates of the cpSec pathway. In contrast, no cpSecA-containing complexes were obtained with the Delta pH pathway substrate tOE23 or the cpSRP pathway substrate LHCP. However, very little crosslinking was achieved for tOE23, possibly because it does not bind thylakoids tightly. Additional experiments were conducted with Delta pH pathway precursors that do bind to the membrane (Figure 2-9, *Panel B*). tOE17 is a truncated form of tOE17, DT-17 and DT-23 are precursors that possess a dual targeting (DT) signal peptide. When the DT signal peptide is fused to a cpSec pathway passenger protein, the resulting precursors are transported on both the cpSec and the Delta pH pathway. However, fusion proteins containing Delta pH

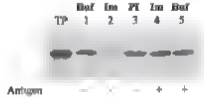


Figure 2-8. rOE33 binding was reduced on anti-cpSecY pre-treated thylakoids. 0.33 µg chlorophyll /µl of thylakoids were pre-incubated with 0.8 µg IgG /µl of anti-cpSecY (C term.) or preimmune in 3% BSA-IBM buffer in the presence or absence of 15 µM cpSecY (C-term.) synthetic peptide. The incubations were for one hour in an ice bath. Thylakoid membranes were washed once with IB buffer and used for rOE33 binding assays as describing in methods. *Lane TP* contains 0.25 µl rOE33 translation product. *Lanes 1-5* contain 6.25 µg chlorophyll of thylakoid membranes from the binding assay samples.

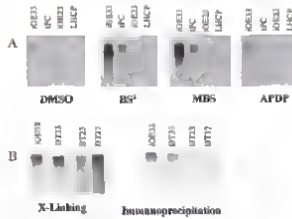


Figure 2-9 Complex formation with different precursors. A. Crosslinking studies with a variety of precursors and crosslinking agents. The anticpSecA immunoprecipitation products of the cross-linking complex from the thylakoid membrane are shown. 1mM cross-linkers BS^3 (Bis [sulfosuccinimidyl] suberate),or MBS (m-Maleimidobenzoyl-N-hydroxysuccinimide ester), or APDP (N-[4-(p-Azidosalicylamido) butyl]-3'-[2'-pyridyldithio] propionamide) were used for the samples in panels as marked DMSO is the solvent used for preparing the cross-linkers MBS. APDP tPC is a truncated form of the pPC precursor and is transported by the cpSec pathway (Henry et al 1997) tOE23 is transported by the Delta pH pathway LHCP is integrated into the thylakoid membrane by the chloroplast SRP pathway B Crosslinking with precursors that bind tightly to thylakoids. tOE17 is a truncated form of tOE17 and is transported exclusively by the Delta pH pathway, DT33 contains a dual targeting signal peptide and is transported by the cpSec and the Delta pH pathway; DT17 also contains the dual targeting signal peptide, but is only transported by the Delta pH pathway Binding assays were as described above and contained apyrase (2 units/75 µl assay) DSS was added to the cross-linking reaction at 1mM. The cross-linking complex formed on the thylakoid membrane and the anticpSecA immunoprecipitation product of the complex are shown in the left and right panels, respectively

passenger proteins, although readily transported by the Delta pH pathway, are not translocated by the cpSec pathway (Henry et al. 1997). In addition, these later proteins bind tightly to the membrane. As shown in *panel B*, total membrane crosslinking products were obtained for tOE17, DT17, DT-23 (not shown), and DT33. However, CpSecA-containing crosslinking products were only obtained for tOE33 and DT-33. Thus, only those precursors that can be transported by the cpSec pathway produced cpSecA-containing complexes.

Discussion

Transport of proteins on the thylakoidal cpSec pathway requires stromal protein, specifically cpSecA, ATP, and is stimulated by a transmembrane ΔpH. Protease treatment of thylakoids eliminates their ability to translocate proteins, indicating that thylakoid membrane bound proteins are also involved. With regards to precursor requirements, a Sec-type signal peptide is essential and a compatible mature protein domain is important for efficient transport. Here we have confirmed previous results of Haward et al. (1997), that cpSec transport can occur in two stages: the first is precursor binding to the membrane and formation of a complex with cpSecA. The second step is translocation across the membrane. We have characterized the binding and translocation steps in some detail, determined at which of these steps the above transport requirements are employed, and examined the stage at which precursor sequences are involved. Our analyses confirm Haward et al.'s (1997) observation that tOE33 formed a productive thylakoid-bound intermediate during the transport when apyrase is present to eliminate ATP from the reaction. Furthermore, our results confirm that the intermediate contains cpSecA. We

show here that cpSecY is also present in the complex, providing the first evidence that cpSecY plays a role in cpSecA-dependent protein transport. cpSec precursor binding to the thylakoid membrane requires cpSecA, and accumulation of the bound precursor is resulted from transport inhibition in the absence of ATP. Subsequent transport of the bound precursor requires ATP, but was not stimulated by additional cpSecA. Precursors capable of being transported by the cpSec pathway were crosslinked into the cpSecA containing complex, whereas precursors transported by the Delta pH pathway and SRP pathways were not.

As noted by Haward et al. (1997), binding does not require ATP, but occurs maximally in its absence. In our experiments, it was necessary to deplete ATP from assay mixture with apyrase to obtain maximum binding to the membrane. We also tried other methods for arresting the transport and promoting precursor binding such as non-hydrolyzable ATP analogues, ionophores, and azide. But so far, apyrase is the only reagent that could promote precursor binding and the complex formation. The apyrase function for arresting precursor transport is presumed to be its ATPase activity that depletes ATP in the reaction. Boiled apyrase lost its function for stimulating precursor binding and the complex formation (data not shown). The effect seen by removing ATP was a specific one because simply inhibiting the transport by dissipating the pH gradient or adding sodium azide were ineffective in promoting binding and had little effect on binding even when ATP was depleted from the assays. Binding of Sec precursor proOmpA to the *E. coli* membrane also occurred in the absence of ATP (Cunningham et al. 1989). Bound pOE33 was transported from the bound state into the lumen upon re-supply of ATP. It is surprising that a ΔpH did not stimulate the chase reaction. This could

explain, in part, the relatively low percentage of bound precursor that is subsequently transported. In a typical transport assay, the ΔpH is responsible for ~70% of the transport of tOE33. In *E. coli*, the last step of proOmpA transport could be achieved by either SecA related ATP hydrolysis or PMF (proton motive force) alone in the absence of ATP and SecA (Schiebel et al. 1991). For unknown reason, it seems that the bound tOE33 could only be chased into thylakoid lumen with cpSecA related ATP hydrolysis. A ΔpH actually reduced the chase because in the presence of ionophores, that eliminated the delta pH, the chase was stimulated. A recent report indicates that PMF facilitates dessertion of SecA from *E. coli* membrane (Nishiyama et al. 1999). A ΔpH might undergo similar function and result in less chase because release of cpSecA from thylakoid membrane.

Chloroplast SecA (cpSecA) facilitates OE33 transport into the thylakoids (Yuan et al. 1994b, Nakai et al. 1994). The fact that most cpSecA is localized in stroma implies that cpSecA functions at early stage of precursor transport which starts in the stroma and ends in the thylakoid lumen. Indeed, cpSecA stimulated precursor binding (Figure 2-2A). The cpSecA was involved in the precursor binding was also suggested by azide treatment which slightly reduced precursor binding and subsequent chase. Azide is a characteristic inhibitor of the ATPase activity of SecA (Oliver et al. 1990). Of interesting is that chase of bound precursor into the thylakoid lumen was not stimulated by soluble cpSecA (Figure 2-4). In *E. coli*, SecA promotes precursor translocation by undergoing ATP-driven cycles of membrane insertion and dessertion. Each cycle of SecA insertion and dessertion drives 20-30 aminoacyl residues of the precursor across the membrane (Economou and Wickner 1994). So, complete translocation of a precursor requires

multiple cycles of SecA insertion and deinsertion. It was suggested that after the deinsertion the membrane bound SecA might exchange with soluble SecA or reenter the insertion cycle. From our work, because soluble cpSecA could not stimulate the chase, it seems that cpSecA engaged in the precursor binding/transport maintains contact with the membrane during the precursor chase. This is consistent with the results of Chen et al. (1996) for *E. coli* SecA. The soluble cpSecA's inability to stimulate the chase also implies that, during transport, cpSecA binds to the thylakoid membrane before precursor does as demonstrated in *E. coli* system by Cunningham et al. (1989).

The bound precursor could be crosslinked into a high molecular weight complex. Since the complex formation required cpSecA and the complex contained cpSecA and cpSecY, it is called cpSec complex. The detection of such a cpSec complex with bound precursor was an indication that the precursor was engaged in the transport machinery. The idea was further supported by the fact that the precursor binding on the thylakoid membrane was mediated by the protein-protein interactions (Figure 2-5). The results are consistent with those regarding *E. coli* SecA, that binding between precursor and SecA is very weak in solution, that a strong complex is formed between precursor and SecA on the membrane, and that that complex also contains cpSecY. My results differed from those of Howard et al. (1997) in two respects. First, they obtained a 1:1 crosslinking product between cpSecA and precursor. My crosslinking product was a large migrating product that contains both cpSecA and cpSecY. For the most of my work, crosslinker DSS was used at 1 mM while they used DSS at 0.1 mM or less. A lower concentration of cross-linker might reserve individual cross-linking products which may merge to a larger product with other components when higher concentration cross-linker was used. The

reason for higher concentration cross-linker usage in our experiments was that I could have higher yield of cross-linking product interested. Hayard et al. had also obtained a large cross-linking product, but may not show it in their immunoprecipitation. Second, they did not find cpSecY in their complex. Characteristics of their antibodies were not reported. It does not know if their antibodies recognize cpSecY during immunoblotting or immunoprecipitation. In this study, all of antibodies raised against cpSecY immunoprecipitate cpSecY *in vitro* translation product. The pea C-terminal antibody also recognizes cpSecY during immunoblotting (Mori and Cline, unpublished)

Presumably, cpSecY functions in thylakoid transport in a manner similar to SecY in bacteria, forming part of the membrane translocon. The present results showing that cpSecY was a component of the crosslinked cpSec complex and provides evidence that cpSecY functions with cpSecA in an analogous manner as the bacterial homologues. Recent results from Mori and Cline (unpublished data) demonstrate that antibodies against cpSecY synthetic peptide inhibit protein transport on the cpSec pathway. The cpSecY antibodies inhibited tOE33 binding, indicating that cpSecY was involved in the binding step. tOE33 could either bind to a component associated with cpSecY or bind directly with cpSecY. The antibodies could reduce the binding by disturbing the indirect or direct association between tOE33 and cpSecY.

To know the cpSec complex size is important because it will give information about the complex composition. From the results shown above, the cpSec complex contains at least cpSecA , cpSecY and precursor. CpSecA may work as a dimer. So, the complex could be at least about 300 kD. When the complex was analyzed with SDS-PAGE, it migrated on the gel in a somehow abnormal way. It remains on the top of the separating

gel when a acrylamide concentration higher than 3.5% was used, and it appeared as a smeared band on a 4-16% gradient gel. Under the same conditions, molecular weight markers (67-669 kD) ran as discrete bands. I do not quite understand the complexes' behavior on the gels. The cross-linked complex may represent a mixture of multiple products. In *E. coli*, the Sec translocase contains more than 9 sub-units (Duong and Wickner 1997). Part of our cross-linking products could contain all the components in the thylakoid transport machine and other parts could contain only several components of the machine.

cpSec complex formation was precursor specific (Figure 2-9). Our analysis shows that precursors destined for the Delta pH translocation system do not enter into the cpSec complex. Even when Delta precursors bind strongly to the membrane and can be crosslinked to thylakoid membrane proteins, they fail to be crosslinked to cpSecA. Of special interest are chimeric precursors that consist of a dual targeting signal peptide linked to a Delta pH passenger protein. These proteins also fail to be crosslinked to cpSecA. Previous studies have shown that the DT signal peptide is capable of directing efficient cpSec transport when coupled to a passenger protein that is normally transported on the thylakoidal Sec pathway, but not when linked to Delta pH passenger proteins (Henry et al. 1997). This suggests that these proteins may not be recognized by cpSecA, possibly because they cannot adopt a compatible loop for the cpSecA system. It has been shown in *E. coli* that the first 20-30 residues of the mature protein sequence play an important role in transport. When unpaired basic residues are introduced into this region, it poisons translocation. Delta pH precursors do contain significant numbers of basic residues in their N-termini. Support for this notion comes from recent experiments that

show that OE23 can be efficiently translocated across the Sec pathway when fused to the pPC transit peptide plus 25 residues of mature domain (Cline, unpublished).

A primary interest in my study was to determine the step or reaction that commits a precursor to the cpSec pathway. SecA or SecY in *E. coli* Sec system are considered to be receptors. Since the first detectable interaction between precursor and components of the cpSec pathway machinery was on the membrane, I assume that recognition occurs on the membrane. The interaction on the membrane could involve primarily cpSecA given the fact that about 20% cpSecA in chloroplasts was on the thylakoid membrane (Yuan et al. 1994). Two lines of evidence from my experiments support the notion that cpSecA on the membrane could function as a receptor. Firstly, cpSecA stimulates precursor binding to the membrane and the bound precursor is associated with cpSecA. Secondly, since soluble cpSecA does not stimulate precursor chase cpSecA must bind to the thylakoid membrane before or at the same time as precursor does, which is consistent with a receptor role. cpSecY is also involved in precursor binding and is part of the cpSec complex, which supports its role as a receptor. DT33, DT23 and DT17 all could be cross-linked to cpSecY (data not shown), but only could DT33 be cross-linked to cpSecA. It is possible that cpSec-compatible precursors can directly bind to cpSecY as ER precursors can directly bind to Sec61, but strong binding and subsequent transport requires binding to cpSecA. Because DT-23 and DT-17 appear to bind cpSecY, but not to cpSecA, then it could be that the block in cpSec transport of these precursors is their inability to bind cpSecA.

In summary, my results are consistent with the model that thylakoidal Sec substrates are committed to the Sec pathway on the membrane and that the mature region of the mature protein as well as the signal peptide plays a critical role in that reaction.

CHAPTER 3
PROTEIN TARGETING AND TRANSLOCATION ON THE DELTA pH PATHWAY

Abstract

The Delta pH pathway is one of two systems for protein transport into the thylakoid lumen. This pathway requires only the trans-thylakoid pH gradient to power translocation. A newly constructed precursor protein (tOE17), that uses the Delta pH pathway, binds to the membrane in substantial amount in the absence of the ΔpH. Binding is productive because bound precursor is transported into the lumen when the ΔpH is restored. Several observations suggest that binding is due to protein-protein interactions. Protease pretreatment of thylakoids reduced their ability to bind precursor; precursor binding was sensitive to salt concentration; and productive binding was competed by saturating amounts of a Delta pH pathway precursor protein. These results suggest that precursor tOE17 binds to components of the Delta pathway translocation machinery. Transport from the bound state, which we refer to as chase, was very efficient: up to 90% of bound precursor could be chased into the thylakoid lumen. Consistent with the transport requirements, the ΔpH was the only energy source necessary for the chase reaction. The identification and characterization of a productively bound intermediate in this study provide biochemical tools for detecting the nature and function of components of the Delta pH pathway. Antibodies raised to *E. coli*-expressed Hcf106, when pre-bound to thylakoids, specifically inhibited transport on the Delta-pH pathway and reduced the

level of productive binding. The results suggest that Hcf106 functions at early steps of the transport process, possibly as a receptor.

Introduction

The Delta pH pathway was first recognized as a distinct transport pathway by the discovery that different subgroups of precursors had different energy and soluble protein requirements for translocation. Specially, transport of precursors OE17 and OE23 required neither soluble protein factors nor NTPs. Their transport depended entirely on the trans-thylakoid ΔpH (Cline et al. 1992). A requirement for specific translocation machinery was further demonstrated by precursor competition studies (Cline et al. 1993). In a competition assay with thylakoids, saturating amounts of tOE23 selectively competed with tOE17 on the same pathway but not with tOE33 on the other pathway.

Although the Delta pH pathway was identified years ago, knowledge about its mechanism is quite limited. One reason is that intermediate steps of process had not been identified. Identification of intermediates in other systems directly led to identification of components. The import of protein across the chloroplast envelope is one excellent example (reviewed by Cline and Henry 1996, Schnell 1998). In the presence of low concentration of NTPs (50-100 μM), protein import into chloroplast is arrested, resulting in bound precursor on the envelope membrane. When the ATP concentration is raised to approximately 1 mM, the bound precursor is translocated into chloroplast. Bound intermediates were then used to identify import machinery components (Schnell and Blobel 1993, Schnell et al. 1994, Kessler et al. 1994, Ma et al. 1996). The ability to arrest productively bound precursor on the thylakoid cpSec pathway (see chapter 1 in this

dissertation and Haward et al. 1997) led to identification of components and precursor elements in the targeting process. Biochemical approaches, such as cross-linking and native gel techniques to identify machinery components have not been successful for the Delta pathway system because intermediates have not been produced.

In this study, certain truncated Delta pH pathway precursors were observed to bind tightly to the thylakoid membrane in the absence of ΔpH, and the bound precursors were transported into the thylakoids after the ΔpH was restored. The characteristics of precursor binding and chase process will be described.

Materials and Methods

Preparation of Radiolabeled tOE17 by *in vitro* Translation

The coding sequence of tOE17 was amplified with the forward primer 5-AATTATGGCGGGCCGCCGCGC-3' and reverse primer 5'-GTTTTCCCAGTCACGAC-3' (Neb1212.Prr) using a pGEM7zf plasmid containing maize pOE17 as a template. The PCR product was restricted with *XbaI* and ligated into *XbaI* and *SmaI* restricted pGEM 4Z. Capped RNA for tOE17 was produced as described by Cline (1988). RNA was translated in a wheat germ system (Cline et al. 1989) in the presence of [^{3}H]-leucine.

Preparation of Other Precursors

In vitro transcription plasmids for tOE33, tOE23 and LHCP were described as Cline et al. (1993). Preparation of tOE23, tOE17, tPSAN, DTPC, DT17, DT23, DT33 were described as Henry et al. (1997). Preparation of HynPC was described as Mori et al. (1998).

Over-expression of tOE17, DT23 and pOE33 in *E. coli*

The coding sequence of tOE17 was amplified with the forward primer 5'-CGTTGCGGGATCCGGCCGCCG-CGCCGTGATCG-3' and reverse primer 5'-CCATTATTAGAATTCGCGTCTAGCCTAGCTTGGCG -3' using the pOE17 plasmid as a template. The PCR product was restricted with *BamHI* and *EcoRI* and ligated into appropriately restricted pGEX-2T plasmid. tOE17 was expressed as a glutathione S – transferase (GST) fusion protein in *E. coli*. Expression, purification and digestion of the fusion protein with thrombin followed the BRL company instructions. The final tOE17 product after thrombin cleavage was two amino acids different from the *in vitro* translation product. Instead of the amino acids MA, *E. coli*-expressed tOE17 has the amino acids GS at its amino terminus. *E. coli*-expressed DT23 and pOE33 were produced as described respectively by Henry et al. (1997) and Cline et al. (1993).

Preparation of Chloroplasts, Lysates, Thylakoids and Strome

Pea chloroplasts were isolated from 9-10-day-old seedling, lysates, thylakoids and stromal extract were prepared from intact chloroplasts as described by Cline (1991).

Thylakoid Protein Transport Assay

Transport assays were conducted in 1.5 ml microcentrifuge tubes. *In vitro* translation product (0.5 to 5 µl) or 0 to 2 µM *E. coli*- expressed precursor was incubated with 12.5 µg chlorophyll of thylakoids or chloroplast lysate in a total volume of 37.5 µl of import buffer (50 mM Hepes buffer pH 8, 0.33 M sorbitol). Transport reaction was conducted in 70 µE $m^{-2}s^{-1}$ white light for 15 minutes at 25°C. After incubation, thylakoids were recovered by centrifugation and washed with import buffer. Final membrane samples

were either analyzed directly on SDS-PAGE or treated with 10 μg thermolysin in 100 μl of import buffer (Cline, 1986) and then analyzed by SDS-PAGE and fluorography

Precursor Binding and Chase Assays

Ten μl [^{3}H] labeled *in vitro* translated precursor was incubated with 25 μg chlorophyll of thylakoids or chloroplast lysate in the presence of 3 μM CCCP or 2 units of apyrase for 15 minute at 0°C in a total volume of 75 μl of import buffer. The thylakoid membranes were then pelleted at 2500x g, washed with import buffer two times, and divided into two equal portions. One portion was directly analyzed to access the amount of precursor bound to the thylakoid membrane. The second portion was incubated under light for 15 minutes at 25°C in a total of 37.5 μl of chase buffer mixture, which contained ~200 μg protein of stromal extract and 1mM DTT. In some assays, thylakoids recovered from binding or chase assays were treated with 10 μg of thermolysin in 100 μl of import buffer (Cline 1986)

Preparation of Thermolysin- or Proteinase K- Treated Thylakoids for use in Binding Assays

Thermolysin-treated thylakoids were prepared as following. 0.5 μg chlorophyll/μl of thylakoids were incubated with 0-160 μg thermolysin/ml from a 2 mg/ml stock containing 10 mM $CaCl_2$. The incubation was in import buffer on ice and lasted for about 60 minutes. The thylakoid membranes were then recovered by centrifugation and washed twice with import buffer containing 10 mM EDTA and once with stromal extract (containing ~2.5 μg protein/μl) in import buffer. The washed thylakoids were then resuspended in import buffer at 1 mg chlorophyll/ml. The proteinase K treated-thylakoids were prepared as following. 0.5 μg chlorophyll/μl of thylakoids were

incubated with 0-80 µg protease K /ml from a 1mg/ml stock. The incubation was in import buffer on ice and lasted for about 60 minutes. PMSF (Phenylmethylsulfonyl Fluoride) was added to 5 mM and the incubation was continued for an additional 10 minutes on ice. Thylakoid membranes were then recovered by centrifugation and washed twice with import buffer containing 2 mM PMSF and once with stromal extract (~2.5 µg/µl) in import buffer. The washed thylakoids were resuspended in import buffer at 1mg chlorophyll/mL

Analysis of Samples

Samples recovered from the assays were subjected to SDS-PAGE and fluorography (Cline 1986). Quantification of the amount of transport, binding or chase was accomplished by scintillation counting of radiolabeled proteins extracted from excised gel bands (Cline 1986).

Results

Dissipating the ΔpH across Thylakoids Arrested tOE17 Transport and Resulted in Accumulation of a Transport Intermediate on the Thylakoid Membrane

Protein transport into the thylakoid lumen is presumed to start with a targeting event in which the precursor interacts with a transport machinery component. For the Delta pathway, precursor targeting undoubtedly occurs on the thylakoid membrane because soluble components are not required for transport. However, before this study, stable precursor binding to the thylakoids had not been observed. I surveyed a number of precursors, both authentic and recombinant, and found several ones that bound to the thylakoid membrane in the absence of ΔpH (see below).

tOE17 is an N-terminal truncated form of tOE17 that I constructed as part of another study on targeting determinants (Henry et al. 1997). All thylakoid precursor proteins contain lumen-targeting domains (LTD) which can be divided into four sub-domains: an acidic domain (A), a charged domain (N), a hydrophobic domain (H), and a cleavage domain (C) for proteolytic processing. tOE17 contains only the N, H and C domains. While the H, C domains and the followed mature part are exactly the same as tOE17, the N domain in the far end of tOE17 contains only MAGRR amino acid residues. According to Henry et al. (1994, 1997), N, H and C domains in the LTD are sufficient for specific precursor targeting and transport. Unexpectedly, truncation of tOE17 produced our most efficient Delta pH pathway transport substrate. Figure 3-1 shows transport assays conducted with tOE17 under varying assay conditions. The assay was started by incubating radiolabeled tOE17 *in vitro* translation product with the purified thylakoids. Upon transport into the thylakoid lumen, the LTD is removed from the precursor, resulting in the smaller mature OE17. Figure 3-1 demonstrates that tOE17 transport was inhibited when the ΔpH across thylakoid membrane was either dissipated by a combination of ionophores, nigericin and valinomycin, or prevented from forming by incubation in the dark with apyrase to deplete ATP. This figure also shows that tOE17 exhibits transport conditions identical to authentic Delta pH pathway precursors, i.e. it occurs in the absence of ATP or stroma, and it was absolutely dependent on the pH gradient.

Although tOE17 transport was inhibited by depleting the ΔpH, a substantial quantity of precursors remained bound to the thylakoid membrane. Bound precursor was degraded by protease treatment of thylakoids, demonstrating that it was exposed on the exterior of

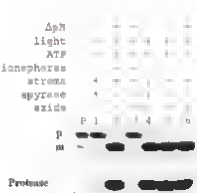


Figure 3-1 tOE17 transport was inhibited by dissipating the ΔpH, resulting in accumulation of bound precursor on the thylakoid membrane. Ten µl [^{3}H] leucine labeled tOE17 precursor was incubated with 25 µg chlorophyll of chloroplast lysate or isolated thylakoids (*lane 5*) in a total of 75 µl of import buffer. The assays were conducted under conditions designed to test the effects, on binding and transport, of removing ATP or stromal extract, of dissipating the ΔpH, or of inhibiting cpSecA with azide. Stromal extract was about 400 µg of protein per 75 µl assay. Mg ATP was added to 5 mM final concentration, the ionophores nigericin and valinomycin were added to 0.5 µM and 1 µM, respectively, from ethanolic stocks. Apyrase treatment was 2 units /75 µl assay. Azide was added to 10 mM final concentration. After incubation at 25°C for 15 minutes the thylakoid membranes were recovered by centrifugation, washed with import buffer two times, and divided into two equal portions in new microfuge tubes. One portion was directly analyzed with SDS-PAGE and fluorography (*top panel*). The second portion was first treated with protease and then analyzed by SDS-PAGE and fluorography (*bottom panel*). Each lane contained 12.5 µg chlorophyll of thylakoid membranes. *Lane P* represents 0.25 µl (5%) precursor added to the assay reaction. The positions of the precursor tOE17 (**p**) and the mature form (**m**) are marked.

the thylakoids. The mature OE17 was resistant to protease treatment confirming its presence in the thylakoid lumen.

Bound Precursor is a Productive Intermediate because it can be Chased into the Lumen when the ΔpH is Restored

If the tOE17 binding is physiologically significant, then bound precursor should proceed into the thylakoid lumen once the ΔpH is restored. I refer to this transport from the bound state as "chase" Binding and chase assays were conducted as shown in Figure 3-2. Radiolabeled precursor was incubated with thylakoids in the dark in the presence of the ionophore CCCP (Carbonylcyanide 3-chlorophenylhydrazone) or with apyrase at 0°C for 15 minutes, during which time tOE17 precursor bound to the thylakoid membrane. Then unbound precursor and ionophore or apyrase were washed away with import buffer and the thylakoids with bound precursor were resuspended in buffer containing stroma and DTT (dithiothreitol) and transferred to 25°C and white light. Under light the bound precursor was chased into the thylakoids. The time required for total chase was ~8 minutes. About 5.6-9.4% of the added precursor to the assay was bound to the thylakoid membrane and ~80% of the bound precursor chased into the lumen. The results indicated that the bound precursor is a productive intermediate on the Delta pH pathway

Other precursors that can be transported on the ΔpH pathway, most of them made in Cline's laboratory, were also tested in the binding and chase assays. As shown in Figure 3-2 (B), the ability to bind tightly to the thylakoid membrane varied among these precursors. tOE17, DT17 and DT23 bound strongly to the thylakoid membrane and a substantial percentage of bound precursor was chased into thylakoids. tOE17 exhibited moderate binding and some bound was chased into thylakoids. HyaPc or tPSAN

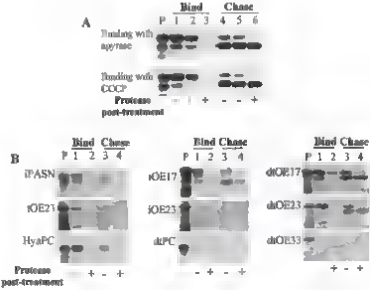


Figure 3-2 Precursor binding-chase experiments. A. Seventy five μg chlorophyll of thylakoids were incubated with 30 μl [^{3}H]- labeled tOE17 *in vitro* translation product in darkness in the presence of 3 μM CCCP or 6 units of apyrase in a total 225 μl of import buffer. The incubation was for about 15 min on ice. The thylakoid membranes were washed with import buffer, divided into 6 portions, and transferred to new tubes. One portion was analyzed directly on SDS-PAGE (*lane 1*). The second and third portions were treated with thermolysin (*lane 3*) or mock buffer (*lane 2*) and then analyzed on SDS-PAGE. The remaining portions were incubated for chase into the lumen. The chase started with the recovered thylakoids from the binding incubation. The thylakoids were resuspended in 37.5 μl IB containing about 180 μg protein of stromal extract and 1mM DTT (dithiothreitol). The resuspended thylakoids were transferred to 70 μE.m^{-2}s^{-1} white light and incubated for 15 minutes at 25°C. Chase samples were then analyzed directly (*lane 4*), or treated with thermolysin (*lane 6*) or mock-buffer (*lane 5*) and then analyzed on SDS-PAGE. Each lane was loaded with 12.5 μg chlorophyll of thylakoids from each assay. *Lane p* represents 0.25 μl of the precursor added to the assay reaction. B. The assays were conducted as described above with a variety of precursors that can be transported on the Delta pH pathway. Binding assays were in the presence of 3 μM CCCP. *Lane 1*: bound precursor on the washed thylakoids; *lane 2*, thermolysin treated binding samples, *lane 3*, chase samples, *lane 4*: thermolysin treated chase samples. *Lane p* represents 0.25 μl of the precursor added to the assay reaction.

exhibited substantial binding but the bound HyaPC and tPSAN were not subsequently chased into the lumen. There was hardly any binding for tOE23, rOE23, DT33 and DTPC. The reason that some precursors can bind better than others is not clear. The binding ability appears to be related to both precursor LTD and the mature protein sequence. More information about these precursors can be found in papers published by Henry et al. (1997) and Mori et al. (1998).

I have examined several ways of dissipating the ΔpH in the dark as a means of accumulating bound precursor. These included apyrase treatment to scavenge all traces of ATP, which could be hydrolyzed by proton-pumping CF_1/CF_0 to generate ΔpH. These also included CCCP treatment. Three μM CCCP was the optimal concentration for arresting the transport and allowing chase. Lower CCCP concentration did not completely arrest transport. Higher CCCP concentration resulted in less binding and did not permit chase. CCCP has the advantage of being water soluble, such that it can be washed from the thylakoid membranes. It is possible that high levels of CCCP cannot be adequately removed by washing or, alternatively, that the high level of CCCP compromises membrane integrity. The ionophore combination of nigericin and valinomycin resulted in high levels of precursor binding, but the bound precursor was not chased. This is consistent with the fact that nigericin and valinomycin are lipid soluble and probably persist though washing steps. Initially, the binding assay was conducted at room temperature. Subsequent tests indicated that binding under icy temperature was comparable to that at room temperature, resulted in a higher percentage of chase. I found it necessary to eliminate the ΔpH, even when binding assays were conducted at 0°C. Because transport of some precursor on the Delta pH pathway is so efficient that it occurs

at icy temperature (Figure 3-3). Precursor binding and subsequent chase in the Delta pH pathway occurred in the presence or absence of stromal extract, so stroma was not required for productive binding (data not shown)

Chase Requirements for the Productively Bound tOE17

The ΔpH across the thylakoid membrane is the only energy requirement for protein transport on the ΔpH pathway. Although I assumed that transport of bound tOE17 occurred on the Delta pH pathway, it was important to examine the chase requirements. A chase requirement assay is reported in Figure 3-4. The chase was inhibited by either nigericin and valinomycin or dark plus apyrase treatment. Apyrase treatment in light did not inhibit the chase. These results indicate that ΔpH is required for the chase. Buffer containing Mg^{++} resulted in higher percentage of chase than buffer alone. As expected, ATP did not stimulate the chase. Stromal extract was not required for the chase, but had some stimulation effect. I also examined the chase requirements for DT-23. This precursor has a dual targeting signal peptide, but is only transported on the Delta pH pathway. Bound DT-23 was also transported exclusively by a Delta pH pathway reaction.

tOE17 Binding is Dee to Protein-Protein Interaction Suggesting thet the Bound Precursor be Associating with Some Machinery Component on the Transport Pethwey

The binding-chase experiment demonstrated that the binding is productive. However, I did not know whether the binding is specific or not. Specific binding should exhibit several characters. (1) the bound precursor should be specifically associated with a specific component, probably a protein component of the transport machinery; (2) the

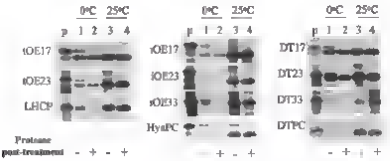


Figure 3-3 Substantial transport could be achieved on the Delta pH pathway at icy temperature for some precursors Nine µl *in vitro* precursor was mixed with 25 µg chlorophyll of chloroplast lysate, 200 µg protein of stromal extract and 5 mM ATP in a total 75 µl of import buffer Incubation was conducted either in icy water (0°C) or 25°C in white light After incubation for 20 minutes, samples were transferred to an ice bath and 0 5 µM/1 µM ionophores nigericin/ valinomycin were added. Thylakoid membranes were recovered by centrifugation, washed with import buffer two times, and divided into two equal portions in new microfuge tubes One portion was directly analyzed with SDS–PAGE and fluorography (*Lanes 1 and 3*). The second portion was first treated with protease and then analyzed by SDS-PAGE and flurography (*Lanes 2 and 4*) Each lane contained 12.5 µg chlorophyll of thylakoid membranes present in each assay *Lane P* represents 0 25 µl precursor added to the assay reaction.

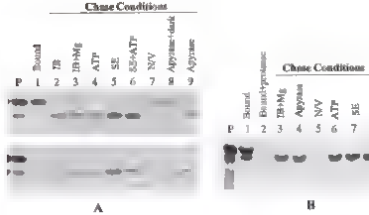
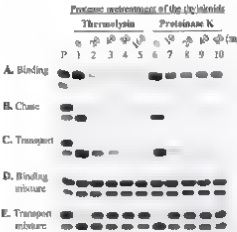
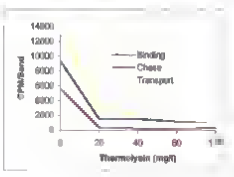


Figure 3-4 Chase requirements for the productively bound tOE17 and DT23. **A.** 225 µg chlorophyll of thylakoids were incubated with 90 µl ^{2}[H]-labeled tOE17 *in vitro* translation product in darkness in the presence of 3 µM CCCP for about 15 min on ice. The thylakoids were recovered by centrifugation and washed one time with import buffer. The washed thylakoids were resuspended in import buffer, divided into 9 equal portions and transferred to new tubes. One portion was used to assess the amount of bound precursor (*lane 1*)— half of the thylakoid membranes was directly resuspended in SDS sample buffer; the other half was treated with protease thermolysin before resuspending in SDS sample buffer. The remaining portions received further chase treatments under conditions designed to test the effects of ATP, ΔpH, stromal extract (SE) and Mg++. Stromal extract was present at about 375 µg protein. Mg++ or Mg-ATP was added to 5 mM final concentration. Nigericin and valinomycin (N/V) were added to 0.5 and 1 µM, respectively, from an ethanolic stock. Apyrase was added at 2 unit/75 µl assay. The chase assay was conducted under 70 µE.m^{-2}s^{-1} white light (darkness for *lane 8*) for 15 min. at 25°C. Finally, half samples were treated with thermolysin (low panel); another half samples were mock treated with import buffer (upper panel). *Lane p* contained an aliquot of the precursor added to the binding incubation. **B.** Experiments were conducted similarly as above but precursor DT23 was used. Samples were treated with thermolysin after the chase.

binding should be saturable, (3). The binding should be competed by precursors known to use the same pathway

I tested if the binding involved a protein-protein interaction (Figure 3-5). tOE17 was incubated with protease-treated thylakoids and assayed for binding or transport. Bound precursor was subsequently analized for chase Before incubation with precursor, the protease-treated thylakoids were thoroughly washed with buffer containing protease inhibitors in order to eliminate any residual protease from the preparation. Precursor binding to the protease-treated thylakoids was substantially reduced compared to control (buffer washed thylakoids). Proteinase K pretreatment had less of an effect on precursor binding than thermolysin treatment. The subsequent chase of any bound precursor on both protease treated thylakoids was totally inhibited indicating that both protease treatments damaged the transport system. This was also demonstrated by assaying the treated membrane for transport with fresh precursor It is of interest that thermolysin effects on binding and transport are highly correlated. The nature of the precursor binding was also assessed by urea and sodium carbonate washes. Proteins that are embedded in the lipid phase of a membrane are not extracted by these wash treatments, whereas extrinsic or peripheral proteins that are associated with membranes via protein-protein interaction are removed (Fujiki et al. 1982) All bound precursors on the thylakoids were extracted by the 0.1M sodium carbonate and most of the bound precursors were extracted with 6M urea washes (data not shown) These results further suggest that the precursors bind directly to membrane proteins under experimental conditions.





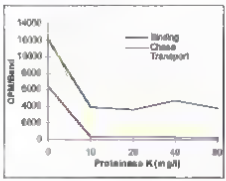


Figure 3.5 tOE17 binding on the thylakoid membrane is mediated by protein-protein interactions. ^{3}H labeled tOE17 *in vitro* translation product was incubated with thermolysin- (0-160 μg/ml) or proteinase K- (0-80 μg/ml) treated thylakoids in the presence of 3 μM CCCP ionophore. Binding, chase and transport assays were conducted essentially as previously described. *Panels A B* and *C* are the buffer washed thylakoid membranes samples after binding, chase and transport assays. To confirm that no residual protease remained in the reactions, aliquots were removed from binding and transport incubation mixtures and analyzed directly on SDS PAGE. As shown in *Panels D* and *E*, free precursors in the solution were not degraded indicating that the pre-protease treated thylakoids did not contain any residual protease

The tOE17 Binding is Salt Sensitive

The transit peptides in Delta pH pathway precursors contain a twin-arginine motif that is crucial for the transport pathway selection (Chaddock et al 1995). One possibility is that the positive charges in the motif may be involved in the interaction between precursor and a putative receptor. If so, then binding should be sensitive to salt concentration. The binding assays were conducted in the presence of increasing concentrations of KCl. As shown in the Figure 3-6, precursor binding in the presence of higher concentration of KCl was decreased as was the subsequent chase. Transport, however, was less influenced by KCl. Transport actually increased slightly from 0-150 mM KCl and decreased at further higher concentration of KCl.

Productive Binding can be Competed by Delta pH Precursors

If the precursor binding on the thylakoid membrane is an interaction between precursor and a receptor-like component, binding should be saturable and competible. To test this, binding of radiolabeled *in vitro* translated tOE17 was conducted in the presence of increasing concentrations of unlabeled *E. coli*-produced precursor proteins (Figure 3-7). The presence of increasing concentration of unlabeled tOE17 precursor resulted in decreased radiolabeled precursor binding, although there remained residual binding that actually increased at high concentration of unlabeled tOE17. Importantly the amount of productively bound precursor decreased dramatically. Productively-bound precursor was determined as that which chased into the lumen upon favorable conditions. From the chase experiment, it is significant that competition for productive binding follows a similar inhibition curves as competition for transport of freshly added precursor. Thus,

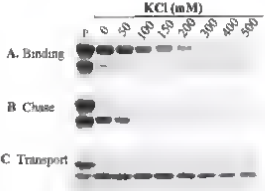


Figure 3-6. tOE17 binding in the presence of increasing concentrations of KCl (0-500mM) *Panel A and panel B* are binding-chase assays. Eight tubes, each of which contained 10 µl ^{35}H-labeled tOE17 *in vitro* translation product and 9 µl import buffer, were preincubated with 1 unit (µl) apyrase for 10 minutes on ice and then mixed with 37.5 µl 0, 100 200 300 400 600 800, or 1000 mM KCl in import buffer Twenty five µg chlorophyll of thylakoids in 17.5µl import buffer which was preincubated with 1 unit apyrase, was added to each tube The final incubation volume of each tube was 75 µl and the KCl concentration was 0-500 mM After 15 minutes incubation on ice the thylakoid membranes in each tube were washed respectively with 0, 50, 100, 150 200, 300, 400, or 500 mM KCl in import buffer and then washed with import buffer The washed thylakoid membranes were divided to 2 equal portions and transferred to new tubes. One portion was analyzed directly on SDS-PAGE for binding (*panel A*) The second portion was incubated for chase. The thylakoids in final chase samples were treated with thermolysin and then analyzed on SDS-PAGE (*panel B*) *Panel C* shows transport f freshly added precursor Chloroplast lysate (12 5 µg chlorophyll) was mixed with 1 5 µl tOE17 translation product in the presence of 0-500 mM KCl in a total 37.5 µl of import buffer *Lane p* represents 0 25 µl of the precursor added to the assay reaction. The other lanes in *panel A, B* and *C* were loaded with 6 25 µg chlorophyll of thylakoids from each assay

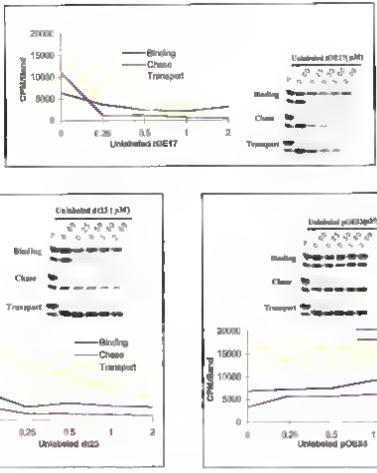


Figure 3-7 Binding competition. Binding-chase assays started with 10 µl ^{3}H-labeled tOE17 *in vitro* translation product and 25 µg chlorophyll of thylakoids in the presence of increasing concentrations of unlabeled *E coli* produced tOE17 or pOE33 or DT23 in a total 75 µl import buffer. Before the adding of radiolabeled precursor, unlabeled precursors were pre-incubated with chloroplast lysate for 3 min on ice. Subsequent chase was conducted with the washed thylakoids after the binding. Results of ^{3}H-OE17 transport in the presence of 0-2 µM *E. coli*-produced unlabeled tOE17 or pOE33 or DT23 were also shown.

there appears to be two kinds of binding involved. One is productive binding, presumably from an interaction between precursor and transport machinery. Another is non-productive or non-specific binding. The amount of non-productive binding varied from experiment to experiment. It became a significant percentage of total binding when *E. coli*-produced precursors were present in the binding assay. This is demonstrated most dramatically in the tOE17 binding saturation experiment (data not shown). Therefor, the non-productive binding may result from precursor aggregation on the thylakoid membrane. It seems apparent to me from Figure 3-7 that the productive binding is competible and the non-productive binding is not. Similar results of competition for labeled tOE17 binding-chase and transport were also obtained with unlabeled DT23 as competitor. pOE33 is transported on the cpSec pathway. *E. coli* produced pOE33 did not have any effects on tOE17 binding, chase or transport. The above results demonstrate that elevated concentration of tOE17 saturates a membrane component involving in the precursor binding process.

In Figure 3-7, it is apparent that in vitro translation of tOE17 produces two products. One is tOE17, a second migrates at the approximately location of mature OE17. This lower band, which varies in amount with the translation reaction, also binds to the membrane. It is unlikely that it is mOE17 because there is only one methionine in tOE17 that could serve as initiator and because it is competed by DT-23 as well as tOE17 but not pOE33, indicating that it is being competed by virtue of the targeting sequence rather than the mature sequence. It is not clear how it is produced from the *in vitro* translation, but may result from early termination, which has previously been observed with wheat germ translation systems (Mori and Cline, unpublished).

Bound Precursor Chase is Competible

We further want to know whether there is any competition for transport from the bound state. The radiolabeled tOE17 was allowed to bind on the thylakoids without competitor. After washing away unbound free precursor the thylakoid membranes with bound precursor were then resuspended in the buffer containing 0 to 2 μM unlabeled tOE17 and a chase reaction was conducted. As shown in Figure 3-8, chase of bound precursor decreased in the presence of unlabeled precursor. The results indicate that in addition to competition for binding the chase reaction can also be competed. We have noticed that a significant amount of bound precursors was chased into the thylakoids even in the presence of high levels of unlabeled competitor. This did not occur in the competition for transport that was conducted as a control with the CCCP treated thylakoids plus freshly added labeled precursor under the same condition as the chase.

tOE17 Binding is Not Reversible

One possibility for the above chase competition is that during the chase process the bound radiolabeled precursor is released into solution and re-enters the Delta pH pathway as a soluble precursor. In this case, the observed competition for chase would be equivalent to competition for transport of free precursors. To clarify this issue, a different binding competition experiment was conducted to assess the reversibility of binding (Figure 3-9). In the above binding competition tests, chemical amount of *E. coli.* produced unlabeled precursors were incubated first with thylakoids and the radiolabeled *in vitro* translation precursors were added afterwards. If radio labeled precursors were

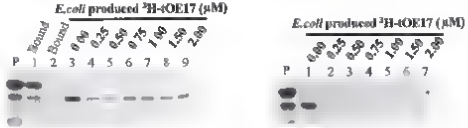


Figure 3-8 Bound precursor chase competition. In the left panel, 112.5 μg chlorophyll of thylakoids were incubated with 45 μl ^{3}H- labeled tOE17 *in vitro* translation product in dark in the presence of 3 μM CCCP. After 15 minutes incubation on ice, the thylakoids were recovered by centrifugation and washed with import buffer and divided into 9 equal portions and transferred to new tubes. For checking precursor binding, one portion was directly analyzed with SDS-PAGE (*lane 1*). second portion was treated with protease thermolysin, and then analyzed with SDS-PAGE (*lane 2*). The thylakoid membranes in the remaining portions (*lane 3-9*) were resuspended in buffer containing increasing concentrations of unlabeled *E coli* produced tOE17 and incubated under chase conditions. Final chase samples were treated with thermolysin. Transport assay controls are presented in the right panel. 12.5 μg chlorophyll of thylakoids, which were pre-treated with 3 μM CCCP and buffer-washed, were mixed with buffer containing 1 μl ^{3}H- labeled tOE17 *in vitro* translation product and increasing concentrations of unlabeled *E coli* produced tOE17 and incubated under chase conditions. Final transport samples were treated with thermolysin. *Lane P* contains an aliquot of the precursor added to assays. The remaining lanes in both panels were loaded with 6.25 μg chlorophyll of thylakoids from each assay.

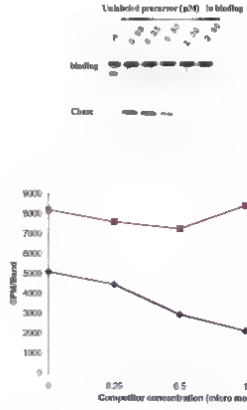


Figure 3-9 Effect of unlabeled precursor on the binding of preloaded radiolabeled tOE17 *in vitro* translation product. Two µl radiolabeled tOE17 was incubated with 25 µg chlorophyll of thylakoids for 5 min, then 0-2 µM unlabeled *E. coli* produced precursor (tOE17) was added and the incubation continued for 15 minutes. After washing with import buffer, the membranes were transfer to new tubes. Half of each sample was analyzed directly for the amount of precursor bound to the membrane; remaining half of each sample was incubated for chase in a buffer containing stroma and DTT

incubated first with the thylakoids, as binding was not competed by the added unlabeled precursor However, the subsequent chase of the bound radiolabeled precursor was decreased. The results demonstrate that the precursor binding is quite strong and hardly replaced. Also, the results further confirm that there is real competition at chase step, though the competition occurs between the bound precursors.

Hcf106 is involved in the tOE17 binding

Hcf106 was identified in maize as a component of the Delta pH pathway by both *in vivo* and *in vitro* methods (Voelker and Barkan 1995, Settles et al. 1997). Tha4 is a maize homologue of Hcf106 a cDNA for psTha4, a pea homologue of Tha4 was recently isolated in Cline's laboratory Antibodies against maize Hcf106 or psTha4 were prepared (Mori et al., in preparation) The effects of antibodies against maize Hcf 106, pea psTha4 and pea cpSecY, on tOE17 binding-chase and transport to pea thylakoids, were examined. Thylakoids were pre-incubated with either antiserum IgG or preimmune serum IgG After washing the thylakoids were assayed for binding-chase and transport of ^{3}H-labeled tOE17

As shown in Figure 3-10, Hcf106 and psTha4 antibodies inhibited tOE17 transport and chase, indicating Hcf106 and psTha4 function on the Delta pH pathway The inhibitions caused by antibody against Hcf106 could be reversed by antigen Hcf106 protein. Antibody against cpSecY, which plays an essential role on the cpSec pathway, did not have any inhibition. I have noticed that although both Hcf106 and psTha4 antibodies specifically inhibited tOE17 transport and chase, only Hcf106 antibody reduced the binding. Preliminary experiments indicated that the bound tOE17 could form

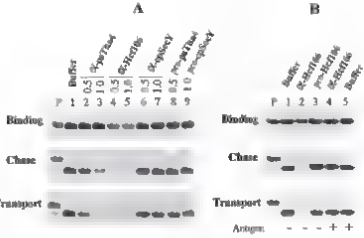


Figure 3-10 tOE17 binding-chase and transport to the thylakoids pre-treated with Hcf106 psTha4 or cpSecY antibodies. In A panels, 0.33 µg chlorophyll /µl of thylakoids were pre-incubated with 0.5 or 1.0 µg IgG /µl of immune or preimmune in 3% BSA-IBM buffer. The incubations were for one hour in an ice bath. Thylakoid membranes were washed once with IB buffer and used for binding-chase and transport assays as describing in methods. In B panels, thylakoids were pre-incubated with 0.8 µg IgG /µl of anti-Hcf106 or preimmune in 3% BSA IBM buffer in the presence or absence of 15 µM Hcf106 antigen. Following steps as A panels. For both A and B panels, *Lane P* contains 0.25 µl tOE17 *in vitro* translation product. The remaining lanes contain 6.25 µg chlorophyll of thylakoid membranes from the assay samples.

a complex with Hcf106. tOE17 was cross-linked on the thylakoid membrane. Anti-Hcf106 prevented the formation of cross-linking products. The crosslinking products were immunoprecipitated with antibody against Hcf106. These results suggest that Hcf106 function in the precursor binding process, possibly as a receptor.

Discussion

For the first time, I show here that a typical Delta pH pathway precursor accumulated on the thylakoid membrane when the ΔpH was dissipated. The bound precursors were transported into the thylakoids when the ΔpH was restored. The construction of precursor tOE17 allows me to successfully demonstrate that transport on the Delta pH pathway occurs in two steps, binding and then translocation. I think that the binding step represents the targeting reaction on the Delta pH pathway. The tOE17 transit peptide only has N, H and C domains with the N domain containing MAGRR residues. Nevertheless, tOE17 is a typical substrate for the Delta pH pathway. Its transport does not require any soluble components from the chloroplast stroma and the ΔpH across the thylakoid membrane is the only energy requirement. Its transport also was competible with native precursor tOE23 from the Delta pH pathway (data not shown) and its transport was totally inhibited by Hcf106 antibody. Of interest, the first precursor observed to exhibit productive binding is DT23. It has a dual targeting transit peptide but only can be transported by the Delta pH pathway (Henry et al. 1997). In the presence of ionophores DT23 transport was inhibited and substantial precursor remained bound to the thylakoid membrane. Because the DT transit peptide can target a cpSec pathway passenger protein transport on both Delta pH and cpSec pathways and can only target a Delta pH pathway

passenger on Delta pH pathway (Henry et al. 1997), it was thought that bound DT23 was stuck in the cpSec machinery. At that time no Delta pH pathway precursor had been found binding tightly to the thylakoid membrane. However, further experiment indicated that the bound DT23 was on the Delta pH pathway and was not stuck in Sec machinery (data not shown). Furthermore the bound DT23 was subsequently transported on the Delta pH pathway and competed by the Delta pH pathway precursor. All the experiments shown above for tOE17 were first tried with DT23 with similar results. We also found that native precursor tOE17 behaved the same in those experiments mentioned above, although the binding was much less. Why tOE17 can bind much tighter than the native precursor on the thylakoid membrane is not clear. All of tightly bound precursors tOE17, DT23 and DT17 have a short transit peptide. However, tOE23 that also has a truncated (short) LTD similar to tOE17 does not bind strongly. The longer transit peptide in native precursor has an A domain which function is unknown.

Precursor binding was sensitive to KCl concentration, indicating that an ionic interaction is involved. All Delta pH pathway precursor contain a twin-arginine motif, which is crucial for targeting the precursor into the pathway (Chaddock et al. 1995). It is highly possible that the RR is used for precursor specific binding. However, without the RR, a truncated precursor named KK17 still bound on the thylakoid membrane, although it was not subsequently chased into the lumen (data not shown). This suggests that RR may not be involved in the binding and its role may be played at translocation step. Besides the twin-arginine, other domains in the transit peptide are also required for the targeting (Chaddock et al. 1995, Henry et al. 1997). Therefore, precursor binding could involve multiple components on the thylakoid membrane. Bound tOE17 could be cross-

linked to thylakoid proteins and the cross-linked complexes could be immunoprecipitated with Hcf106 antibody. Comparing the cross-linking and immunoprecipitation products from bound tOE17 and KK17 may give insight into whether or not RR specifically interacts with some components on the thylakoid membrane.

The binding competition tests in Figure 3-7 demonstrated that the binding was competible, implying that a saturable component of the transport machinery is involved in binding. However, when binding was conducted with increasing concentrations of ^{3}H-labeled *E. coli* produced tOE17 the total binding increased linearly with higher precursor usage. While the binding of *E. coli*-produced tOE17 is not saturable the subsequent chase exhibited saturation. The direct transport also saturable and displayed the same kinetics (data not shown). The chase competition was unexpected. It is not likely but possible that under "chase conditions" the bound precursor is released into the solution and then re-enters the transport pathway. If this is true, then transport of bound precursor cannot be considered to be chased from an intermediate step. The chase is considered to be a process in which the bound precursor directly transfers to the translocon from the binding site and is translocated into the lumen. In this case, the observed chase competition would actually be competition for a full transport reaction. Several methods, including conducting chase under different volumes of buffer, were tested to determine whether the chase was achieved directly. The results were inconclusive. However, we noticed that unlike the transport competition control a significant amount of bound precursor transported into the thylakoids regardless of how high the concentration of competitor was (Figure 3-8). This is evidence that the observed chase is different from the normal transport. The binding is considered to be an interaction between precursor and a

receptor. The subsequent chase should be directly achieved by shift of the bound precursor from the receptor to an associated translocon. Then, any competition occurring at this step must result from multiple receptors sharing a common translocon. Preliminary crosslinking data from Cline's laboratory (Mori and Cline, unpublished data) indicates that Hcf106 exists in thylakoids as a multimer. If Hcf106 functions as a receptor, competition for chase may reflect the existence of an Hcf106 multimer that serves as some sort of antenna receptor complex.

The Delta pH pathway is a very novel system considering that the ΔpH across thylakoids is the only energy requirement. It was originally thought to be a unique pathway of chloroplasts. Now its homologues have been found in bacteria (Settles and Martienssen 1998). Before this study, no Delta pH pathway precursors were found to bind tightly to the thylakoid membrane, making identification and purification of components very difficult. The binding-chase techniques developed in this study should provide an important tool to isolate machinery components and help understand the transport mechanism.

CHAPTER 4
SUMMARY AND CONCLUSIONS

The first part of my research is about protein transport on the cpSec pathway. Advancing the understanding about the mechanism is achieved by dissecting the transport process into targeting and translocation stages and the related characterization of each stage. Productive binding with precursors to the thylakoid membrane was stimulated with cpSecA and occurred when transport was inhibited by removing ATP from the assay with apyrase. cpSecA may function as a determinant for transport pathway selection as only the precursors using the cpSec pathway can form an complex with it. cpSecY was found to be another machinery component associated with bound precursor, providing the first evidence that cpSecY is functioning on cpSec transport pathway. Subsequent translocation of bound precursor required ATP, but was not stimulated by additional cpSecA. cpSecA had been speculated to be the Sec pathway receptor, given the fact that it is the only soluble component required for the transport on the cpSec pathway. It would be logical that cpSecA interacts with precursor in the stroma and targets the precursor to the translocon in the thylakoid membrane. However, no such interaction was detected. Our results support the notion that precursor commitment to the thylakoid cpSec pathway occurs on the membrane, rather than in the soluble stroma. Since the first detectable interaction between precursor and components of the cpSec pathway machinery was on the membrane, we assume that the receptor residences on the membrane.

The second part of my research is about protein transport on the Delta pH pathway. This pathway is unique for its special energy requirement and has been a recent focus of research in thylakoid protein transport. However, for years, knowledge about the mechanisms involved in this system has been quite limited. A biochemical approach such as cross-linking and native gel techniques to identify the machinery components had been difficult for the system since the routinely used precursors did not bind tightly to the thylakoid membrane. A newly constructed precursor protein tOE17 allows me to develop the technique of dissecting the whole transport into binding (targeting) and chase (translocation) steps. tOE17 binds to the membrane in substantial amount in the absence of the ΔpH and the bound precursor is chased into the lumen when the ΔpH is restored. Several observations suggest that binding is due to protein-protein interaction. Productive binding was competed by saturating amounts of a ΔpH pathway precursor protein, suggesting that machinery components from the Delta pH pathway are involved. Consisting with the transport requirement, the ΔpH was the only energy source used for chase. Interestingly, chase of the bound precursor could be competed with unlabeled precursor. Hcf106 and Tha4 are two components identified recently. Both maize Hcf106 antibody and pea Tha4 antibody specifically inhibited tOE17 transport and chase to the pea thylakoids. However, only antibody raised against Hcf106 reduced tOE17 binding to the thylakoid membranes. Given the fact that most part of the protein is facing the stroma, Hcf106 may function as a receptor.

REFERENCES

Abad MS, Clark SE, Lamppa GK. 1989 Properties of a chloroplast enzyme that cleaves the chlorophyll a/b binding protein precursor *Plant Physiol.* 90:117–24

Akita M, Sasaki S, Matsuyama S, Mizushima S 1990. SecA interacts with secretory proteins by recognizing the positive charge at the amino terminus of the signal peptide in *E. coli*. *J Biol Chem* 265:8164–69.

Akiyama Y, Ito K. 1985 The SecY membrane component of the bacterial protein export machinery analysis by new electrophoretic methods for integral membrane proteins. *EMBO J* 4:3351–56

Anderson CM, Gray J 1991 Cleavage of the precursor of pea chloroplast cytochrome f by leader peptidase from *Escherichia coli* *FEBS Lett* 280:383–86.

Bassham DC, Bartling D Mould RM, Dunbar B, Wenbeek F, Herrmann RG Robinson C 1991 Transport of proteins into chloroplasts Delineation of envelope "transit" and thylakoid "transfer" signals within the pre-sequences of three imported thylakoid lumen proteins. *J Biol Chem.* 266:23606–10

Berghöser J, Karnauchov I, Herrmann RG, Klösgen RB 1995 Isolation and characterization of a cDNA encoding the secA protein from spinach chloroplasts. *J Biol Chem.* 270:18341–46

Bogsch E, Brink S, Robinson C 1997 Pathway specificity for a delta pH-dependent precursor thylakoid lumen protein is governed by a 'Sec-avoidance' motif in the transfer peptide and a 'Sec-incompatible' mature protein. *EMBO J* 16:3851–59

Bogsch EG, Sargent F, Stanley NR, Berks BC, Robinson C, Palmer T 1998 An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. *J Biol Chem* 273:18003–06

Brundage L, Hendrick J, Schiebel E, Driessen A, Wickner W 1990 The purified *E. Coli* integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. *Cell* 62:649–657

Caliebe A, Grimm R, Kaiser G Lübeck. J, Soll J, Heins L. 1997 The chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding protein. *EMBO J* 716:7342–7350

Clark SA, Theg SM. 1997 A folded protein can be transported across the chloroplast envelope and thylakoid membranes. *Mol. Biol. Cell* 8:923–34

Chaddock AM, Mant A, Karnauchov I, Brink S, Herrmann RG, Klosgen RB, Robinson C. 1995 A new type of signal peptide: central role of a twin-arginine motif in transfer signals for the delta-pH-dependent thylakoidal protein translocase. *EMBO J* 14:2715–22.

Chen X, Xu H, Tai PC. 1996 A significant fraction of functional SecA is permanently embedded in the membrane: SecA cycling on and off the membrane is not essential during protein translocation. *J Biol. Chem.* 271 29698-29706.

Chua N-H, Schmidt GW. 1978 Post-translational transport into intact chloroplasts of a precursor to the small subunit of riburose-1, 5-bisphosphate carboxylase. *Proc. Natl. Acad. Sci. USA* 75 6110-6114

Chua N-H, Schmidt GW. 1979 Transport of proteins into mitochondria and chloroplasts. *J. Cell Biol.* 81:461-483

Chitnis PR, Nechushtai R, Thornber JP. 1987 Insertion of the precursor of the light-harvesting chlorophyll a/b-protein into the thylakoids requires the presence of a developmentally regulated stromal factor. *Plant Mol. Biol.* 10:3-11

Cline K, Werner-Washburne M, Lubben TH, Keegstra K. 1985 Precursors to two nuclear-encoded chloroplast proteins bind to the outer envelope membrane before being imported into chloroplasts. *J. Biol. Chem.* 260:3691–96

Cline, K. 1986 Import of proteins into chloroplasts: Membrane integration of a thylakoid precursor protein reconstituted in chloroplast lysates. *J. Biol. Chem.* 261:14804-14810[Abstract]

Cline K, Fulsom DR, Viitanen PV. 1989 An imported thylakoid protein accumulates in the stroma when insertion into thylakoids is inhibited. *J. Biol. Chem.* 264:14225–32.

Cline K, Ettinger WF, Theg SM. 1992 Protein-specific energy requirements for protein transport across or into thylakoid membranes. *J. Biol. Chem.* 267:2688–96.

Cline K, Henry R, Li CJ, Yuan JG. 1993 Multiple pathways for protein transport into or across the thylakoid membrane. *EMBO J.* 12:4105–14

Cline K, Henry R. 1996 Import and routing of nucleus-encoded chloroplast proteins. *Annu. Rev. Cell Dev. Biol.* 12:1–26

Creighton AM, Hulford A, Mant A, Robinson D, Robinson C. 1995 A monomeric, tightly folded stromal intermediate on the delta pH-dependent thylakoid protein transport pathway. *J. Biol. Chem.* 270:1663–69

Cunningham K, Lill R, Crooke E, Rice M, Moore K, Wickner W, Oliver D 1989 SecA protein, a peripheral protein of the *Escherichia coli* plasma membrane is essential for the functional binding and translocation of proOmpA. *EMBO J* 8:955–959

de Boer AD, Weisbeek PJ 1991 Chloroplast protein topogenesis: import, sorting and assembly *Biochim. Biophys Acta* 1071: 221–53

Dobberstein B, Blobel G, Chua, N-H 1977 In vitro synthesis and processing of a putative precursor for the small subunit of ribulose-1,5-bisphosphate carboxylase of Chlamydomonas reinhardtii. *Proc Natl Acad Sci. USA* 74:1082-1085

Duong F, Wickner W 1997 Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme *EMBO J* 16:2756-68

Economou A, Wickner W 1994 SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell* 78 835-843

Ento T Kawakami M, Gogo A, America T, Weisbeek P, NaKai M. 1994 Chloroplast protein import. chloroplast envelopes and thylakoids have different abilities to unfold proteins *Eur J Biochem.* 225 403-409

Files JD, Bassford PJ Jr 1989 Novel secA alleles improve export of maltose-binding protein synthesized with a defective signal peptide. *J Bacteriol* 171 402-409

Fincher V, McCaffery M, Cline K. 1998 Evidence for a loop mechanism of protein transport by the thylakoid Delta pH pathway *FEBS Letters* 423:66-70

Friedman AL, Keegstra K. 1989 Chloroplast protein import quantitative analysis of precursor binding *Plant Physiol* 89 993–99

Haehnel W, Jansen T, Gause K, Klosgen RB, Stahl B, et al. 1994 Electron transfer from plastocyanin to photosystem I *EMBO J* 1 1028–38

Hageman J, Baecke C, Ebskamp M, Pilon R, Smeekens S, Weisbeek P 1990 Protein import into and sorting inside the chloroplast are independent processes *Plant Cell* 2 479–94

Halpin C, Elderfield PD, James HE, Zimmerman R, Dunbar B–Robinson C. 1989 The reaction specificities of the thylakoidal processing peptidase and Escherichia coli leader peptidase are identical. *EMBO J* 8 3917–21

Hand JM, Szabo LJ, Vasconcelos AC Cashmore AR. 1989 The transit peptide of a chloroplast thylakoid membrane protein is functionally equivalent to a stromal-targeting sequence. *EMBO J* 8 3195-3206

Hartl F, Lecker S, Schiebel E, Hendrick J, Wickner W. 1990. The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. Coli* plasma membrane. *Cell* 63:269-279

Haward SR, Napier JA, Gray JC. 1997. Chloroplast SecA functions as a membrane-associated component of the Sec-like protein translocase of pea chloroplasts. *European Journal of Biochemistry* 248:724-730

Henry R, Carrigan M, McCaffery M, Ma XY, Cline K. 1997. Targeting determinants and proposed evolutionary basis for the Sec and delta-pH protein transport systems to chloroplast thylakoid membranes. *J. Cell Biol.* 136:823–32.

Hunaik SC, Hill K, Wagner R, Schleicher T, Soll J. 1997. Reconstitution of a chloroplast protein import channel. *EMBO J.* 16:7351-7360.

Hirsch S, Muckel E, Heemeyer F, von Heijne G, Soll J. 1994. A receptor component of the chloroplast protein translocation machinery. *Science* 266:1989–92.

Hoffman NE, Franklin AE. 1994. Evidence for a stromal GTP requirement for the integration of a chlorophyll a/b-binding polypeptide into thylakoid membranes. *Plant Physiol.* 105:295-304

Hulford A, Hazell L, Mould RM, Robinson C. 1994. Two distinct mechanisms for the translocation of proteins across the thylakoid membrane, one requiring the presence of a stromal protein factor and nucleotide triphosphates. *J. Biol. Chem.* 269:3251-56.

Hynds PJ, Robinson D, Robinson C. 1998. The Sec-independent twin-arginine translocation system can transport both tightly folded and malfolded proteins across the thylakoid membrane. *J. Biol. Chem.* 273:34868-34874

Keegstra K, Cline K. 1999. Protein import and routing systems of chloroplasts. *Plant Cell.* In press.

Kessler F, Blobel G, Patel HA, Schnell DJ. 1994. Identification of two GTP-binding proteins in the chloroplast protein import machinery. *Science* 266:1035–39

Kessler F, Blobel G. 1996. Interaction of the protein import and folding machineries in the chloroplast. *Proc. Natl. Acad. Sci. USA* 93:7684–89

Kim SJ, Robinson D, Robinson C. 1996. An Arabidopsis thaliana cDNA encoding PS II-X, a 4.1 kDa component of photosystem II, a bipartite presequence mediates SecA/delta pH-independent targeting into thylakoids. *FEBS Lett.* 390:175–78

Kirwin PM, Meadows JW, Shackleton JB, Musgrove JE, Elderfield PD, et al. 1989. ATP-dependent import of a lumenal protein by isolated thylakoid vesicles. *EMBO J.* 8:2251–55

Klimyuk, VI, Persello-Cartieaux F, Havaux M, Contard-David P, Schuenemann D, Meiherhoff K, Gouet P, Jones JDG, Hoffman NE, Nussaume L 1999 A chromodomain protein encoded by the arabidopsis CAO gene is a plant specific component of the chloroplast signal recognition particle pathway that is involved in LHCP targeting *Plant Cell*, 11 87-99

Knott TG, Robinson C 1994 The secA inhibitor, azide, reversibly blocks the translocation of a subset of proteins across the chloroplast thylakoid membrane *J Biol Chem.* 26911 7843–46

Ko K, Cashmore AR. 1989. Targeting of proteins to the thylakoid lumen by the bipartite transit peptide of the 33 kd oxygen-evolving protein. *EMBO J.* 8 3187–94

Kouranov A, Schnell DJ 1997 Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. *J Cell Biol.* 139 1677-1685

Kuhn A, Kiefer D, Kehne C, Zhu H-Y, Tschantz WR, Dalbey RE. 1994. Evidence for a loop-like insertion mechanism of pro-Omp A into the inner membrane of *Escherichia coli*. *Eur J Biochem.* 226.891 897

Laidler V, Chaddock AM, Knott TG, Walker D, Robinson C. 1995 A SecY homolog in *Arabadopsis thaliana*. *J Biol. Chem* 270 17664-17667

Lamppa GK. 1988. The Chlorophyll a/b-binding protein inserts into the thylakoids independent of its cognate transit peptide *J Biol. Chem.* 263 14996-14999

Lorkovic ZJ Schröer WP Pakrasi HB, Irrgang KD Herrmann RG Oelmüller R. 1995 Molecular characterization of PsbW a nuclear-encoded component of the photosystem II reaction center complex in spinach. *Proc Natl Acad. Sci USA* 92 8930-34

Ma YK, Kouranov A, LaSala SE, Schnell DJ 1996 Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope *J Cell Biol.* 134 315–27

Meadows JW, Robinson C 1991 The full precursor of the 33-kDa oxygen-evolving complex protein of wheat is exported by Escherichia coli and processed to the mature size *Plant Mol. Biol.* 17 1241–43

Meyer T H, Ménétret J F Breitling R, Miller K R, Akey CW Rapoport T A. 1999 The bacterial SecY/E translocation complex forms channel like structures similar to those of the eukaryotic Sec61p complex. *J Mol Biol* 285 1789-800

Michl D, Robinson C, Shackleton JB, Herrmann RG, Klösgen RB. 1994. Targeting of proteins to the thylakoids by bipartite presequences: CFoII is imported by a novel, third pathway. *EMBO J.* 13:1310–17

Mizushima S, Tokuda H. 1990. In vitro translocation of bacterial secretory proteins and energy requirements. *J. Bioenerg. Biomembr.* 22:389–99. Review

Mori H, Cline K. 1998. A signal peptide that directs non-Sec transport in bacteria also directs efficient and exclusive transport on the thylakoid Delta pH pathway. *J. Biol. Chem.* 273:11405–11408

Mori H, Summer L, Ma X, Cline K. 1999. Component specificity for the thylakoidal Sec and DeltapH-dependent protein transport pathways. (To be submitted)

Morin XK, Soll J. 1997. Immunogold labelling of cryosectioned pea chloroplasts and initial localization of the proteins associated with the protein import machinery. *Planta* 201:119–127

Nilsson R, Brunner J, Hoffman NE, van Wijk KJ. 1999. Interactions of ribosome nascent chain complexes of the chloroplast-encoded D1 thylakoid membrane protein with cpSRP54. *EMBO J.* 18:733–742

Nishiyama K-I, Fukuda A, Morita K, Tokuda H. 1999. Membrane domersion of SecA underlying proton motive force-dependent stimulation of protein translocation. *EMBO J.* 18:1049–1058.

Nohara T, Asai T, Nakai M, Sugiura M, Ende T. 1994. Cytochrome f encoded by the chloroplast genome is imported into thylakoids via the SecA-dependent pathway. *Biochem. Biophys. Res. Commun.* 224:474–78

Oliver DB, Cabelli RJ, Dolan KM, Jarosik GP. 1990. Azide-resistant mutants of Escherichia coli alter the SecA protein, an azide-sensitive component of the protein export machinery. *Proc. Natl. Acad. Sci. USA* 87:8227–31

Olsen LJ, Keegstra K. 1992. The binding of precursor proteins to chloroplasts requires nucleoside triphosphates in the intermembrane space. *J. Biol. Chem.* 267:433–39

Osborne RS, Silhavy TJ. 1993. PrlA suppressor mutations cluster in regions corresponding to three distinct topological domains. *EMBO J.* 12:3391–3398

Payan LA, Cline K. 1991. A stromal protein factor maintains the solubility and insertion competence of an imported thylakoid membrane protein. *J. Cell Biol.* 112:603–13

Pain D, Blobel G. 1987. Protein import in chloroplasts requires a chloroplast ATPase. *Proc. Natl. Acad. Sci. USA* 84:3288–92

Perry SE, Keegstra K. 1994 Envelope membrane proteins that interact with chloroplastic precursor proteins. *Plant Cell* 6:93–105

Pilon M, De Boer AD, Knols SL, Koppelman MHGM, Van der Graaf RM, De Kruijff B Weisbeek PJ 1990 Expression in Escherichia coli and purification of a translocation-competent precursor of the chloroplast protein ferredoxin J Biol. Chem. 265 3358-3361

Pugsley A.1993 The complete general secretory pathway in gram-negative bacteria *Microbiol Rev* 57:50-108

Puzsz JW, Fikes JD Bassford PJ Jr 1989 A analysis of mutational alterations in the hydrophilic segment of the maltose-binding protein signal peptide. *J Bacteriol.* 171:2302-2311

Rapoport TA, Jungnuckel B, Kutay U 1996. Protein Transport Across the Eukaryotic Endoplasmic Reticulum and Bacterial Inner Membranes *Annu. Rev Biochem.* 65 271-303

Robinson C, Ellis RJ 1984 Transport of proteins into chloroplasts: partial purification of a chloroplast protease involved in the processing of imported precursor polypeptides. *Eur J Biochem* 142:337–42.

Robinson C, Klösgen RB. 1994 Targeting of proteins into and across the thylakoid membrane a multitude of mechanisms. *Plant Mol Biol* 26 15–24

Robinson D Karnauchov I, Herrmann RG, Klösgen RB, Robinson C 1996 Protease-sensitive thylakoidal import machinery for the Sec , delta pH- and signal recognition particle-dependent protein targeting pathways, but not for CF_0I integration. *Plant J* 10:149–55

Robinson C, Hynds PJ, Robinson D, Mant A. 1998 Multiple pathways for the targeting of thylakoid proteins in chloroplasts. *Plant Molecular Biology* 38:209-221

Sargent F, Bogsch EG, Stanley NR, Wexler M, Robinson C, Berks BC, Palmer T 1998 Overlapping functions of components of a bacterial Sec-independent protein export pathway *EMBO J* 17 3640-3650

Shaw AS, Rottier PJM, Rose JK. 1988 Evidence for the loop model of signal-sequence insertion into the endoplasmic reticulum. *Proc Natl Acad. Sci USA* 85 7592 7596

Schiebel E, Driessen AJM, Hartl F-U, Wickner W 1991 $\Delta\mu H^+$ and ATP function at different Steps of the catalytic cycle of preprotein translocase. *Cell* 64:927-939

Schnell DJ, Blobel G 1993 Identification of intermediates in the pathway of protein import into chloroplasts and their localization to envelop contact sites. *J Cell Biol.* 120 103–15

Schnell DJ, Kessler F, Blobel G 1994 Isolation of components of the chloroplast protein import machinery *Science* 266:1007–12.

Schnell DJ 1998 Protein targeting to the thylakoid membrane. *Annu. Rev Plant Physiol Plant Mol. Biol.* 49:97–126.

Schuenemann D Gupta S, Persello-Cartieaux F, Klimyuk VI, Jones JDG, Nussaume L, Hoffman NE 1998. A novel signal recognition particle targets light harvesting proteins to the thylakoid membranes. *Proc. Natl. Acad. Sci. USA* 95 10312-10316

Settles AM, Yonetani A, Baron A, Bush DR, Cline K, Martienssen R. 1997 Sec-independent protein translocation by the Maize Hcf106 Protein. Science 278:1467-1470

Settles AM, Martienssen R. 1998 Old and new pathways of protein export in chloroplasts and bacteria. *Trends Cell Biol.* 8:494-501 Review

Seidler A, Michel H. 1990. Expression in Escherichia coli of the psbO gene encoding the 33 kD protein of the oxygen-evolving complex from spinach. *EMBO J* 9:1743–48

Smeekens S, Bauerle C, Hageman J, Keegstra K, Weisbeek P. 1986. The role of the transit peptide in the routing of precursors toward different chloroplast compartments. *Cell* 46:365-75

Soll J, Tien R. 1998 Protein translocation into and across the chloroplastic envelop membranes. *Plant Molecular Biology* 38:191-207

Sugiura M, Hirose T, Sugita M. 1998 Evolution and mechanism of translation in chloroplasts. *Annu. Rev Genet* 32:437-459

Swidersky UE et al. 1992. Biochemical analysis of the biogenesis and function of the Escherichia coli export factor SecY *Eur J Biochem* 207:803-811

Swidersky UE, Hoffsebulte HK, Muller M. 1990 Determinants of membrane-targeting and transmembrane translocation during bacterial protein export. *EMBO J* 9:1777-1785

Theg SM, Bauerle C, Olsen LJ, Selman BR, Keegstra K. 1989 Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplastic membranes. *J Biol. Chem.* 264:6730–36.

Tranel PJ, Froehlich J, Goyal A, Keegstra K. 1995. A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. *EMBO J* 14:2436–46

VanderVere PS, Bennett TM, Oblong JE, Lamppa GK. 1995 A chloroplast processing enzyme involved in precursor maturation shares a zinc-binding motif with a recently recognized family of metalloendopeptidases. *Proc. Natl. Acad. Sci USA* 92:7177–81

Viitanen PV, Doran ER, Dunsmuir P 1988 What is the role of the transit peptide in thylakoid integration of the light-harvesting chlorophyll a/b protein? *J Biol Chem* 263 15000-15007

Voelker R, Barkan A 1995 Two nuclear mutations disrupt distinct pathways for targeting proteins to the chloroplast thylakoid. *EMBO J* 14 3905–14

Voelker R, Mendel-Hartvig J, Barkan A 1997 Transposon-disruption of a maize nuclear gene, tha1, encoding a chloroplast SecA homologue: in vivo role of cp-SecA in thylakoid protein targeting *Genetics* 145 467–78

von Heijne G, Steppuhn J, Herrmann RG 1989 Domain structure of mitochondrial and chloroplast targeting peptides. *Eur J Biochem* 180 535–45

von Heijne G, Nishikawa K 1991 Chloroplast transit peptides. the perfect random coil? *FEBS Lett* 278(1) 1–3

Waegemann K, Paulsen H, Soll J 1990. Translocation of proteins into isolated chloroplasts requires cytosolic factors to obtain import competence. *FEBS Lett* 261 89-92.

Watanabe P, Blobel G 1989 Site-specific antibodies against the PrlA (SecY) protein of Escherichia coli inhibit protein export by interfering with plasma membrane binding of preproteins. *Proc Natl Acad Sci USA* 86 1895-1899

Wenbeek P- Hageman J De Boer D Pilon R. Smeekens S 1989 Import of proteins into the chloroplast lumen. *J Cell Sci* Suppl. 11 199-223

Weiner JH, Bilous PT, Shaw GM, Lubitz SP, Frost L, Thomas GH, Cole JA, Turner RJ 1998 A novel and ubiquitous system for membrane targeting and secretion of cofactor containing proteins. *Cell* 93-93-101.

Wexler Margaret, Bogsch Erik G Klösgen Ralf Bernd, Palmer Tracy, Robinson Colin, Berks Ben C 1998. Targeting signals for a bacterial Sec-independent export system direct plant thylakoid import by the ΔpH pathway. *Febs Letters* 431.339-342

Yuan J, Cline K. 1994a. Plastocyanin and the 33-kDa subunit of the oxygen-evolving complex are transported into thylakoids with similar requirements as predicted from pathway specificity *J Biol. Chem* 269-18463–67

Yuan J, Henry R, McCaffery M, Cline K. 1994b. SecA homolog in protein transport within chloroplasts: evidence for endosymbiont-derived sorting. *Science* 266 796–98

Yuan J, Henry R, Cline K. 1993 Stromal factor plays an essential role in protein integration into thylakoids that cannot be replaced by unfolding or by heat shock protein Hsp70 *Proc Natl Acad Sci. USA* 90 8552–56

Zak E, Sokolenko A, Unterholzner G, Altschmied L, Herrmann RG 1997 On the mode of integration of plastid-encoded components of the cytochrome bf complex into thylakoid membranes. *Planta* 201:334–41

BIOGRAPHICAL SKETCH

Xianyue Ma was born to a big family on March 12, 1958, in Wuhan City, China. He completed high school in 1975. After working for three years in the fields on a farm, he continued his education at the Agriculture University of Central China where he received his bachelor's and master's degrees in crop sciences. In 1985, he was offered a position in Hubei University, where his main duty was teaching plant physiology to undergraduate students. In 1988, Hubei University sent him to England as a visiting researcher. He worked on a photoinhibition project in photosynthesis in Professor James Barber's laboratory for one year. He remained at Hubei University until 1992, when he came to United States to visit his wife. In order to gain laboratory experience in U.S., he worked for Dr. Daryl Prung at the University of Florida as an OPS. His jobs ranged from washing glassware to isolating mitochondria RNA. Xianyue Ma began his Ph.D. study in Dr. Kenneth Cline's laboratory in the fall of 1993, and he received his Ph. D. in May 1999.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

Kenneth C. Cline, Chair
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Alice Harmon
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

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May 1999

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